VARIATION IN THE CHEMICAL COMPOSITION OF GRASS

The carotene and sulphur-amino acids in four species of herbage.

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INTRODUCTION

THE IMPORTANCE OF GRASS. Grass is one of the most widely grown crops and constitutes the basis of feeding stuffs for live stock. From the economic point of view, the farmer's cheapest feeding stuff for his live stock is to be found growing within reach of his home stead. The cultivation of grass will answer this demand.

Grass can be utilized either directly by grazing and soiling or indirectly after being preserved. Hay-making is probably the oldest method for preserving grass. It has long been and is still being practised on the farm. The method of preserving feeds by ensilage has a history of about one hundred years. It has the advantage over grass drying that considerably more land can be dealt with with much less loss of nutrients. Recently the artificial drying method has been introduced for preserving grass. Conservation by artificial drying is superior to natural drying and to ensilage as a method of preserving young grass in that there is practically no loss of nutrients nor deterioration in quality. The improvement in the utilization of grass increases its value and enhances its importance as a feeding stuff.

THE CHEMICAL COMPOSITION OF GRASS. To ensure the best result from grass it is advisable to study its chemical composition. In its biochemical aspects the grass problem is a very complex one. Most studies in crop production and crop quality are concerned with the yield and composition of a certain part of the plant, grain or root, at maturity. What we require of grass is not necessarily high yield of mature material but rather a steady yield of immature material that is of high digestibility and nutritive value. Grass varies in composition from that of a food like a high protein concentrate to one which is but little more nutritious than straw, depending on the stage of maturity, species of grass, fertility of soil, and other conditions including season, climate, etc. The aim of the study of its chemical composition is to enable the farmer to get high yield as well as good quality from grass.

OBJECT OF PRESENT INVESTIGATION. The food requirements of animals are of two kinds. They require certain specific nutrients for body maintenance and growth, and a source of energy to carry on vital processes and reactions. Thus carbohydrates and fats or oils are required for supplying energy; and into the first group fall the proteins contributing vital amino acids,

the mineral constituents, and vitamins. Animals can usually get a sufficient supply of energy-supplying constituents and minerals which together comprise by far the greatest proportions of feeds; but they often lack sufficient amounts of the proteins and vitamins, which are of great importance in animal life.

Up to the present, of the amino acids which are the building stones of the proteins and indispensable to animal life, cystine and methionine are the only two containing sulphur. Before the discovery of methionine, many workers (Willcock and Hopkins, 1907; Osborne and Mendel, 1915; Johns and Finks, 1920, and Sherman and Merrill, 1925) proved that cystine is essential to animals. Jackson and Block (1931, 1932) showed that methionine is also an essential amino caid. Recently, Womack, Kemmerer and Rose (1937) found that methionine is an indispensable amino acid and that normal growth of the animal can be attained in the absence of cystine provided methionine be present. Although it is impossible, on the evidence available at present, to draw a final conclusion as regards the dietary requirement of animals with respect to these two amino acids, it is safe to say that both cystine and methionine must be the principal sources of the

various sulphur compounds of the animal body.

Vitamin A is one of the most important vitamins that must be present in the food of the animal for health, growth, or good production. Grass, whether fresh or artificially dried, is almost the only source of carotene, the precusor of vitamin A, and it is a matter of considerable importance that the farmer should be informed as to its carotene content, and of the means whereby he may control it.

Although much work done in recent years on forage crops has been concerned with proteins and vitamins, information on the influence of different factors on these constituents is still very limited.

With these facts in view, the present investigation aimed at examining the effects of different factors on these important constituents of grass so that its value to the animal may be assessed and the consequences of particular management practices determined. Therefore in this investigation, the relative contents of carotene and sulphur-containing amino acids in several important species of grass, cut at different stages of growth and grown under different manurial treatments, were determined. Besides these, the water content, total nitrogen and

amino-N were also estimated in order to show whether there were any significant correlations.

REVEIW OF LITERATURE

PROTEIN. The presence of glutinous matter, i.e. protein, in other parts than the seed of various plants was first announced by Rouelle (1773). He considered it to be the nutritive substance from which the caseous part of milk was derived. Later Fourcroy (1789) gave an extensive account of the occurrence of coagulable protein in various parts of many plants.

Grindley, Joseph, and Slater (1915) were the first to publish data on quantitative determinations of amino acids in feeds. At about the same time, Mollan (1915) published results on the amino acid contents of certain commercial feeds. Both investigations made use of the Van Slyke method (1911-1912) and difficulties were encountered through the presence of non-protein nitrogenous constituents and the carbohydrates in feeds. Eckstein and Grindley (1919) made some improvement on the old method by removing some of the non-protein nitrogenous constituents with ether and cold absolute alcohol and by converting the insoluble carbohydrates into soluble ones by boiling with 0.1% HCl. Hamilton, Nevens, and Grindley (1921) made a similar study on the amino acid contents of oats, corn, cottonseed meal and alfalfa by the

Van Slyke method (1911, 1912, 1915) and overcame the difficulties by introducing these procedures: (a) the non-protein nitrogenous constituents were removed by extraction with ether, alcohol and 1.0% trichloroacetic acid; (b) the starch was removed by a hot 2.0% trichloroacetic acid extraction. Morris (1934) modified the method used by Hamilton et al. (1921) by determining the amino acids of various feeds after the extraction, by various solvents, of the non-protein nitrogen, fats, carbohydrates and minerals.

However, it was Osborne and Wakeman (1920) who first made a serious attempt to prepare proteins from spinach leaves by the addition of alcohol to the leaf extract and then made some investigations on the nature and nitrogen distribution of the proteins. Osborne, Wakeman and Leaveworth (1921) made a further study on the proteins of the alfalfa plant. Fresh green alfalfa was ground thoroughly and the contents of its cells were extracted by water, alcohol, dilute aqueous alkali, and hot alkaline alcohol, applied in the order named. The amounts of dry matter, ash-free solid, nitrogen, and inorganic constituents in different extracts were determined.

Chibnall and Schryver (1921) extracted proteins

from cabbage and scarlet runner beans and found that ether-water was the most suitable cytolytic agent. The distribution of nitrogen was also determined and agreed well with those reported by Osborne and Wakeman. Since then Chibnall and his collaborators have done much work on the study of leaf protein. Chibnall (1924) separated the so called "combined" and "soluble" proteins by filtrating through paper pulp. The "soluble" proteins of a large number of different leaves have been investigated by Chibnall and Gorver (1926). They suggested that these proteins were glutalins and their cheminal properties were very similar, having an isoelectric range from pH 4.0-5.0. They also found that the H-ion concentration of the leaf cell sap of various plants was in all cases alkaline with respect to their isoelectric range.

Miller and Chibnall (1932) made more amino acid analysis by the method of Van Slyke with cocksfoot protein prepared by using ether-water instead of ether. The amino-acid composition was very similar to that of other leaf proteins extracted from spinach (Chibnall, 1924) and runner beans (Chibnall and Grover, 1926), but the presence of cystine could not be determined. In addition, they also determined the dicarboxylic

acid-N by Damodaran's method (1931). Pollard and Chibnall (1934) prepared some pasture plant proteins by substituting "used" ether-water for "fresh" ether-water and determined their cystine content by Prunty's modification (1933) of Sullivan's colorimetric method. They found that all grass proteins contain cystine varying from 0.8 to 0.95% and that lucerne leaf protein is particularly rich in cystine and contains 1.2%.

Miller (1935, 1) analysed the proteins from a number of typical forage grasses for the basic amino acids by individual isolation of the latter. One year later, Miller (1936) isolated glutamic acid, aspartic acid and proline from cocksfoot protein.

Recently, several investigations have been made by Lugg on the composition of leaf proteins. Different methods for determining sulphur distribution, particularly in their bearing upon the estimation of cystine, cysteine and methionine, were examined and applied to several leaf proteins by him (1938, 1). He pointed out that the various procedures gave the same value in the case of edestin, and that the values obtained with impure leaf proteins varied with the procedure. The extremely low RSSR content of the HCl hydrolysate of cocksfoot protein recalled similar

low cystine contents reported by Pollard and Chibnall (1934). According to Lugg's result, cystine and/or cysteine contents varied from 1.1 to 1.7%, and methionine from 1.2 to 1.6% of the protein-N. At the same time Lugg (1938, 2) also made some amino acid (including tyrosine, tryptophane and sulphur-containing acids) and amide analyses of protein preparations from the fresh leaves of various plants and found that the range over all the leaf protein preparations were: 4.70-5.98% amide-N, 2.09-2.74% tyrosine-N, 1.43-1.98% tryptophane-N, 0.99-1.70% cystine and/or cysteine-N, and 1.01-1.69% methionine-N. Samples of protein extracted from plant leaves in various ways were examined for representativeness and some analyses of the whole proteins of leaves were carried out by Lugg (1939). He pointed out that the amide, tyrosine and tryptophane contents and the sulphur distribution of the whole proteins of leaves provided no evidence of variation in composition with the age of the leaves or the manurial and climatic conditions or locality of growth; but may vary with species.

CAROTENE. It was not until 1913 that extensive studies of the chlorophyll content of leaves were carried out by Willstätter and Stoll (1913). They devised methods

of extracting carotene and xanthophyll. Most subsequent processes are based upon their procedure. Chibnall and Channon (1929) determined the carotenoids in cabbage leaf by Willstätter's method.

The principal constituent of most leaf carotenes has been shown to be the β-isomeride by Kuhn, Winterstein and Lederer (1931), Miller (1935, 2), Mackinney (1935), and Strain (1935). They showed that in many grasses the β-isomeride is the only form that can be detected. Pollard (1936) isolated carotene and xanthophyll from cocksfoot and stated that the carotene appears to consist entirely of the β-isomeride.

The effect of the curing process upon the carotene content of grass has been investigated by several workers (Russell, 1929; Hauge and Aitkenhead, 1931; and Hathaway et al. 1932). They all reported that artificially dried grass had two to seven times as much vitamin A potency as that dried in the field. Russell (1934) made a further investigation of the carotene content of freshly-cut, machine-dried, and field-dried alfalfa, and found that the carotene content of machine-dried alfalfa is not less than that of fresh-cut material from the same field, that machine-dried alfalfa has a higher carotene content than field-

cured, and that the degree of destruction of carotene is determined by the length and condition of exposure in the field.

Some investigations have been made on the loss of carotene from feeding stuffs during storage. Fraps and Treichler (1933, 1) reported an appreciable diminution of vitamin A potency in alfalfa, yellow corn and other materials during storage. Guilbert (1935) showed that the carotene in alfalfa decresed from 30 to 50% during storage for eight weeks at room temperature, while at from -5° to 0°C there was practically no destruction in the same length of time. Bauman and Steenbock (1933) have published results on the stability of carotene in vegetable oils, esters and organic solvents, and found that refined cotton-seed oil is outstanding among the vegetable oils. They also found that carotene is relatively stable in ethyl acetate, ethyl succinate, ethyl alcohol and methyl alcohol; and that all other oils and solvents are markedly inferior, but the loss of carotene can be reduced by the addition of hydroquinone, by replacing the air with nitrogen, or by adding anti-oxidant-containing material like wheat germ oil. McDonald (1933) reported similar results namely that

maize oil and Wesson oil keep carotene better than other oils and that carotene kept in the dark or at a low temperature lasts longer. Turner (1934) reported that carotene dissolved in olive oil loses part of its activity in the course of 12 to 17 months, even in the presence of organic stabilizers. Fraps and Kemmerer (1937) found that the carotene in alfalfa meal was more stable when the meal was stored at 6°C than it was when stored at room temperature, which agreed well with the report given by Guilbert. They also found that alfalfa leaf meal diluted 1 to 9 with corn starch lost carotene more rapidly than it did when it was not diluted.

As to the manurial effect on the carotene content, investigations have been carried out with various crops including grass. Guthrie (1929) worked with soya bean and showed that an insufficient supply of nitrate nitrogen adversely affected the amount of carotenoids. Virtanen et al. (1933) found that the production of carotene is directly affected by the nitrogen available and that nitrates are superior to ammonium salts. They also found that the optimum pH for maximum carotene production in wheat and pea was 6.5. The effectiveness of nitrogen was confirmed by

Ijdo (1936), who also found that potassium salts reacted adversely on the carotene content, and that calcium and magnesium salts and phosphates have little effect. Thomas and Moon (1938) reported that ammonium sulphate produced a marked increase in the carotene content of grass, that ferrous sulphate was without effect, and that calcium carbonate, which was more than sufficient to satisfy the lime requirement of the soil, produced only a small increase in carotene content. Moon (1939, 4) made a further investigation on the influence of manurial treatment on the carotene content of poor pasture grass and found that ammonium sulphate and sodium nitrate produced an increase of 28% in the carotene content, that potassium sulphate produced an increase of 6.2%, and that calcium carbonate produced no effect.

Concerning the effect of age at cutting on the carotene content, Virtanen et al. (1933) found that, with wheat and pea, active carotene formation occurred in the young plant and reached a maximum at the time of flowering; thereafter a very marked decline was observed. They stated that a similar decline occurs in grass. Thomas and Moon (1938) reported that the carotene content does not vary substantially during

a growth period of four weeks and the yield of carotene is more or less directly related to the amounts of dry matter produced. Moon (1939, 1) carried out some experiments on the carotene content in 1938, and reported that the drought in April depressed the carotene content, which remained constant thereafter until flowering began, when a marked loss occurred, but did not further decrease during the post-flowering period.

With regard to the carotene content of different species, Miller (1935, 2) determined the carotenoids, carotene and xanthophyll, in thirteen plant tissues and found that β-carotene contents varied from 31.7 to 140.0 p.p.m. and that the ratio of xanthophyll to β-carotene ranged from 2.42 to 5.55. Atkeson, Peterson and Aldous (1937) have reported the carotene content of several pasture plant commonly grown in Kansas and stated that the carotene content varied with species as well as with the local meteorological conditions. Moon (1939, 2) made an examination of the carotene contents of seven grasses and three clovers at several periods during the growing season and at various stages of growth, and found that clovers are usually not so stemmy as the grasses and have higher carotene contents.

The correlation of carotene to organic and mineral constituents has also been studied recently. Virtanen (1936) has stated that the carotene content of plants is closely related to growth, factors that retard growth affecting carotene content adversely, and optimum growth occurring simultaneously with maximum carotene content. Watson (1937) reported that the carotene content is closely related to the crude protein. Thomas and Moon (1938) examined forty-six samples of grass and concluded that the correlation between carotene and crude protein is highly significant and positive. Moon (1939, 1) also found that the correlation between carotene and the ratio of true to crude protein, and between carotene and fibre are significantly negative. More recently Moon (1939, 4) has shown that the carotene content is closely associated with protein content under manurial treatments which have little or no effect on these two constituents, and under manurial treatments which produce increases in carotene or protein the correlation between them is not so close.

OTHER CONSTITUENTS. Ether-soluble substances, including calcium salts of glycerodiphosphoric acids, fatty acids, etc., of leaf cytoplasm have been studied by

Chibnall and Channon (Chibnall and Channon, 1927, 1, 2, 3; 1929; Channon and Chibnall, 1927, 1929).

Elliot, Orr, and Wood (1926) investigated the mineral content of pasture grass and its effect on herbivora.

The study of carbohydrates has been somewhat neglected, perhaps because the necessary analytical methods have not been available. Norman (1936) undertook some analyses on nitrogen-free extracts in addition to ash, crude protein, crude fibre and ether-soluble material determinations on rye-grass. Norman and Richardson (1937) made a more detailed investigation of water-soluble fructosan of rye-grass. They found that fructosan increased to a peak of 30.1% at about the time of full heading, thereafter decreasing with maturity. Norman (1939, 1) made a similar study with cocksfoot and found that, unlike rye-grass, the water-soluble fructosan in the immature cocksfoot was depressed by the application of nitrogen, whilst the mature sample still contained an appreciable amount of fructosan.

Norman (1939, 2) stated that lignin is almost wholly unavailable to herbivorous animals and that the presence of lignin retards and prevents the bacterial degradation of the cellulose.

MATERIAL AND GENERAL ARRANGEMENT OF INVESTIGATION

MATERIAL. For the present investigation four of the most common species of forage crops were selected:

(1) White clover, Trifolium repens;

(2) Danish Cocksfoot, Dactylis glomerata;

(3) Ayrshire Perennial Rye-grass, Lolium perenne;

(4) Scotch Timothy, Phleum pratense.

During the course of the investigation, fifty three samples were obtained, from several farms, at various stages of growth, and under different local conditions for a study of the effects of species, location, stage of growth, and season, forty being taken in 1939 and the rest being taken in 1940. The descriptions of these samples are given in Table I (see page 19). In addition to these, ten samples of Rye-grass and Cocksfoot grown under different manurial treatments were also collected in the second year (1940) in order to examine the effect of fertilizers. Table III (see page 24) shows the manurial treatments and other conditions of these samples.

Since the chemical composition of grass varies more with the stage of growth than with the date or season of cutting, all the samples were roughly classified as "young", "flowering" or "heading",

"ripe", "old", "young aftermath", "old aftermath", etc., according to the condition of the samples.

Table I.

Sample No.	Species	Source	Date of cutting	Weather at cutting	Stages of growth and remarks
1	White clover	Boghall	12,6,39	Fine	Young
2	Cocksfoot	"	"	"	Heading
3	Rye-grass	"	"	"	Young, a few leaves left
4	Timothy	"	"	"	Heading
5	White clover	Carnwath	14,6,39	Rainy	Very young
6	Rye-grass	"	"	"	Heading
7	Timothy	"	"	"	Young, some decayed leaves
8	White clover	Cause-wayend	27,6,39	Cloudy	Flowering
9	Cocks-foot	Boll-o-bere	"	"	Fully ripe
10	Rye-grass	Cause-wayend	"	"	Heading
11	White clover	Dryden-mains	13,7,39	"	Flowering
12	Cocks-foot	"	"	"	Ripe
13	Rye-grass	"	"	"	Heading
14	White clover	Boghall	20,7,39	"	Flowering

15	Cocks-foot	Boghall	20,7,39	Cloudy	Fully ripe
16	Rye-grass	"	"	"	Fully ripe
17	Timothy	"	"	"	Ripe
18	White clover	"	31,7,39	Rainy	Fading
19	"	Carnwath	3,8,39	"	Fading
20	Cocks-foot	"	"	"	Aftermath, some dead grass
21	Rye-grass	"	"	"	Old, a few seeds left
22	"	"	"	"	Aftermath, some old stems
23	Timothy	"	"	"	Aftermath, some old stems
24	Cocks-foot	Boll-ó-bere	10,8,39	"	Aftermath, some dead grass
25	Rye-grass	Cause-wayend	"	"	Aftermath, some old stems
26	White clover	White moss	"	"	Young
27	"	Dryden-mains	17,8,39	Fine, dry	Old, some old flower stems
28	Cocksfoot	"	"	"	Aftermath
29	Rye-grass	"	"	"	Young aftermath, some old stems

30	White clover	Boghall	18,9,39	Cloudy	Aftermath
31	Cocksfoot	"	"	"	Young aftermath, grazed
32	Timothy	"	"	"	Young aftermath, grazed
33	Cocksfoot	Boll-o-bere	21,9,39	"	Old aftermath
34	Rye-grass	Causewayend	"	"	Aftermath
35	White clover	Carnwath	28,9,39	Fine	Aftermath
36	Cocksfoot	"	"	"	Old aftermath
37	Rye-grass	"	"	"	Aftermath, grazed
38	Cocksfoot	Drydenmains	4,10,39	Rainy	Old aftermath
39	Rye-grass	"	"	"	Aftermath
40	"	Boghall	11,10,39	"	Young aftermath, grazed
41	Cocksfoot	Knightsridge	31,5,40	Cloudy	Young
42	"	"	"	"	Heading
43	"	Newburgh	1,6,40	Fine	Heading
44	Rye-grass	Boghall (Hayfield)	10,6,40	"	Heading
45	"	Boghall (Demonstration plot)	"	"	Heading, grazed

various amounts of slag. All the fertilizers were applied in July, 1939 and grasses were sown about the same time. The different treatments, each replicated four times, were as follows:

Table II.

Block No.	Treatment No.	Treatment (cwt. per acre)			
		CaO			
		Applied as $Ca(OH)_2$	In slag	Total	Slag
I	1	0	2½	2½	10
"	2	10	"	12½	"
"	3	20	"	22½	"
"	4	40	"	42½	"
II	1	5	0	5	0
"	2	3¾	1¼	5	5
"	3	2½	2½	5	10
"	4	0	5	5	20

To ensure the greatest possible differences in the effect of fertilizers, samples were taken from treatment 1 and 4 in Block I. In Block II, the grasses in those plots, which received no slag, did not grow well enough to justify sampling, and samples were collected from treatment 2 and 4, to which the slag was applied at the rate of 5 cwt. and 20 cwt. per acre respectively.

At Boghall, an experiment on the top dressing of hay with $(NH_4)_2SO_4$ was laid down; Rye-grass was predominant. Four plots of dimensions 19'X20' were used for this experiment. Two plots were treated with $(NH_4)_2SO_4$ on the first of June (1940) at the rate of one cwt. per acre, and the other two received no fertiliser. The soil was in a high state of fertility and the whole field had received a liberal dressing of a mixed fertiliser in the early spring.

Table III.

Sample No.	Species	Source	Date of cutting	Weather at cutting	Stage of growth	Manurial treatment (cwt./acre)
48	Cocksfoot	Harrysmuir	17,6,40	Fine, dry	Heading	5 CaO 5 slag
49	"	"	"	"	"	5 CaO 20 slag
50	Rye-grass	"	19,6,40	"	"	5 CaO 5 slag
51	"	"	"	"	"	5 CaO 20 slag
52	Cocksfoot	"	"	"	"	2½ CaO 10 slag
53	"	"	"	"	"	42½ CaO 10 slag
54	Rye-grass	"	20,6,40	"	"	2½ CaO 10 slag
55	"	"	"	"	"	42½ CaO 10 slag
56	"	Boghall	24,6,40	Cloudy	"	0 $(NH_4)_2SO_4$
57	"	"	"	"	"	1 $(NH_4)_2SO_4$

GENERAL ARRANGEMENT OF INVESTIGATION. The samples, weighing about one pound each, were cut at about one inch above ground level with a knife, and were carried to the laboratory in rubber-lined bags with the least possible delay. On arrival in the laboratory, the mixed weeds, dead stems and leaves were removed by hand-picking.

After being thoroughly mixed, a sub-sample of 60-70 g. was taken from each sample at random and cut into fine pieces with scissors. From the finely cut fresh sample, 5 g. were used for estimating carotene and 50 g. were used for the determination of water-content. When the grass was at flowering or heading, the carotene-contents of the flowers or heads, and those of the leaves and stems were determined separately in samples of 5 g. each. The remainder of the fresh samples was weighed and used for extracting protein.

The dried samples, after the moisture determination, were powdered by grinding in a high speed mill and passed through a 64-mesh sieve. The total N-content in these ground samples was then determined.

The proteins extracted from the fresh grasses

were kept in glass tubes sealed with wax for subsequent determinations. The procedure of extraction will be described later. A portion of each of the extracted proteins was used for determining nitrogen-content. From the weight of protein extracted and its nitrogen-content, the extent of extraction was calculated. In additon, the amino-nitrogen-content, sulphur-containing amino acids and total sulphur of the proteins were also determined.

All the determinations were carried out in duplicate and the procedures adopted will be described in the following sections.

ANALYSIS OF SOIL SAMPLES. In order to show the effect of local conditions, soil samples were taken from the various farms where grass samples were collected, and subjected to analysis for pH, and available K_2O and P_2O_5. The pH value was measured by a potentiometer, using a quinhydrone electrode; the available K_2O was determined by the aspergillus method; and the available P_2O_5 by Kirsanov's acid extraction method. The results are shown in Table IV.

The K_2O figures below 0.3 indicate a deficiency of available potassium in the soil; figures above 0.45 a plentiful supply.

The P_2O_5 figures below 5 indicate a deficiency of available phosphate in the soil; figures above 15 a plentiful supply.

Table IV.

Sample No.	Source	Date of sampling	pH	K_2O	P_2O_5	Grass samples collected
1	Boghall	18,9,39	5.90	0.43	12.5	1, 14, 18, 30
2	"	"	7.30	0.43	21.4	2, 15, 31, 58, 61
3	"	"	6.10	0.35	12.5	3, 16, 40, 45, 47, 60, 62
4	"	"	7.10	0.22	20.0	4, 17, 32, 46, 59, 63
5	Causewayend	21,9,39	7.04	0.22	13.6	8, 10, 25, 34
6	Boll-o-bere	"	7.83	0.28	3.7	9, 24, 33
7	Carnwath	28,9,39	4.20	0.39	Trace	5, 6, 19, 20, 21, 22, 35, 36, 37
8	"	"	4.50	0.55	5.4	7, 28
9	Drydenmains	4,10,39	5.40	0.41	7.1	11, 12, 13, 27, 28, 29, 38, 39
10	White moss	24,11,39	5.90	0.27	4.0	26
11	Newburgh	4,12,39	5.20	0.40	5.0	43
12	xHarrysmuir	7,12,39	5.00	0.45	Trace	48, 49, 50, 51, 52, 53, 54, 55
13	Boghall	29,3,40	7.10	0.39	15,00	44, 56, 57
14	Knightsridge	20,5,40	5.20	0.30	6.00	41, 42

X Average of all plots before treatment.

WATER CONTENT AND TOTAL NITROGEN OF GRASSES

METHODS OF ESTIMATION. (a) WATER CONTENT.-Fifty g. of fresh grass were weighed out from each sample at random and dried for 24 hours in an electric oven at 100°C.

(b) TOTAL NITROGEN-CONTENT.-The total nitrogen-content of the dried grass was determined by the Kjeldahl method. One g. of each of the finely ground samples was introduced into a Kjeldahl flask, to which 25 ml. of concentrated H_2SO_4 (free from nitrogen) and about 6 g. of a mixture containing 0.2 g. of selenium (Ashton, 1936) and 5.8 g. of potassium sulphate were added. The mixture was oxidized by boiling on a sand bath. The liquid became clear after 15 to 20 minutes boiling, and "after-boil" lasted about 2 hours making a total digestion period of approximately 2½ hours which, according to Ashton (1936), is essential to complete the conversion of nitrogen in the grass to $(NH_4)_2SO_4$. After digestion, the clear solution was cooled and transferred to a distilling flask by washing 3 or 4 times with distilled water. The liquid was made alkaline with 100 ml. of caustic soda, containing a small quantity of zinc dust, and the ammonia was distilled into a N/10 H_2SO_4 by boiling

PARTICULARS OF GRASS SAMPLES

Sample No. W.C.	C.	R.	T.	Date of cutting	Locality	Stage of growth and remarks W.C.	C.	R.	T.
1				12,6,39	1	Young			
14				20,7,39	1	Flowering			
18				31,7,39	1	Fading			
30				18,9,39	1	Aftermath			
5		6		14,6,39	7	Very young		Heading	
19	20	21		3,8,39	7	Fading	Aftermath, some dead grass	Old, a few seeds left	
		22		"	7			Aftermath, some old stems	
35	36	37		28,9,39	7	Aftermath	Old aftermath	Aftermath, grazed	
8		10		27,6,39	5	Flowering		Heading	
		25		10,8,39	5			Aftermath, some old stems	
		34		21,9,39	5			Aftermath	
11	12	13		13,7,39	9	Flowering	Ripe	Heading	
27	28	29		17,8,39	9	Old, some old flower stems	Aftermath	Young aftermath, some old stems	
	38	39		4,10,39	9		Old aftermath	Aftermath	
26				10,8,39	10	Young			
	2			12,6,39	2		Heading		
	15			20,7,39	2		Fully ripe		
	31			18,9,39	2		Young aftermath, grazed		
	58			1,7,40	2		Heading		
	61			4,9,40	2		Young aftermath, grazed		
	9			27,6,39	6		Fully ripe		
	24			10,8,39	6		Aftermath, some dead grass		
	33			21,9,39	6		Old aftermath		
	41			31,5,40	14		Young		
	42			"	14		Heading		
	43			1,6,40	11		Heading		
		3		12,6,39	3			Young, a few leaves left	
		16		20,7,39	3			Fully ripe	
		40		11,10,39	3			Young aftermath, grazed	
47		45		10,6,40	3	Young, grazed		Heading, grazed	
60		62		4,9,40	3	Young aftermath, grazed		Young aftermath, grazed	
		44		10,6,40	13			Heading	
			4	12,6,39	4				Heading
			17	20,7,39	4				Ripe
			32	18,9,39	4				Young aftermath, grazed
			46	10,6,40	4				Young, grazed
			59	1,7,40	4				Heading
			63	4,9,40	4				Aftermath stalks
			7	14,6,39	8				Young, some decayed leaves
			23	3,8,39	8				Aftermath, some old stems

Table V. Summary of water and nitrogen contents.

Water content (%) W.C.	C.	R.	T.	Total N (%) W.C.	C.	R.	T.
81.40				3.68			
83.40				2.63			
81.65				2.43			
80.60				3.91			
85.08		69.78		4.51		0.95	
86.40	79.30	61.70		2.63	1.42	0.57	
		72.00				1.41	
89.00	76.57	74.09		3.87	1.81	2.01	
83.50		72.70		3.15		1.01	
		72.14				1.22	
		76.90				2.09	
79.87	60.37	61.30		3.03	0.83	1.03	
67.62	66.80	61.00		2.86	1.25	1.36	
	68.00	69.80			1.01	1.84	
82.34				3.82			
	75.42				2.10		
	69.30				0.89		
	84.00				3.98		
	67.50				1.44		
	76.20				2.27		
	69.00				1.22		
	81.40				1.63		
	79.60				1.72		
	79.00				2.53		
	76.60				2.30		
	82.00				1.96		
		70.25				1.31	
		67.50				0.71	
		74.80				4.28	
77.50		72.40		2.99		2.50	
81.25		74.25		3.87		3.07	
		74.00				1.69	
			77.01				2.10
			71.00				1.32
			78.40				2.75
			76.00				3.10
			67.30				1.60
			73.00				1.97
			70.86				1.05
			77.50				1.19

PARTICULARS OF GRASS SAMPLES UNDER DIFFERENT MANURIAL TREATMENTS.

Sample No.	Date of cutting	Locality	Stage of growth	Manurial treatment (cwt. per acre)
Cocksfoot				
48	17,6,40	12	Heading	5 cwt. CaO 5 " slag
49	"	"	"	5 " CaO 20 " slag
Rye-grass				
50	19,6,40	"	"	5 " CaO 5 " slag
51	"	"	"	5 " CaO 20 " slag
Cocksfoot				
52	"	"	"	2½ " CaO 10 " slag
53	"	"	"	42½ " CaO 10 " slag
Rye-grass				
54	20,6,40	"	"	2½ " CaO 10 " slag
55	"	"	"	42½ " CaO 10 " slag
Rye-grass				
56	24,6,40	14	"	0 $(NH_4)_2SO_4$
57	"	"	"	1 cwt. "

Table VI. Summary of water and N contents of grasses under different manurial treatments.

Water content (%)	Total N (%)
Cocksfoot	
68.00	0.96
69.90	0.89
Rye-grass	
65.60	0.54
66.60	0.69
Cocksfoot	
64.60	0.82
63.60	0.66
Rye-grass	
66.80	0.86
67.60	0.72
Rye-grass	
64.40	0.71
66.40	1.03

for half an hour. The acid in excess was titrated with N/10 NaOH using methyl red as an indicator. The calculated nitrogen-content was expressed as "% of dry matter".

RESULTS AND DISCUSSION. The water and nitrogen contents of all samples are given in Table V and Table VI. For the convenience of examining the effect of different factors, the particulars of each sample, which have been listed in Table I and Table III, are also given in the attached tables which will be referred to for the subsequent tables. The figures under the column "locality" refer to the soil sample number (see Table IV page 27). Grass samples taken from the same localtiy are grouped together and arranged according to the time of cutting so that the variation in chemical composition can be better compared. In the same table and some subsequent tables, W.C. stands for White clover, C. for Cocksfoot, R. for Rye-grass, and T. for Timothy.

It is obvious that the moisture content is not of great importance in discussing the results, because it depends largely upon the climatic conditions prevailing when the samples are taken. There are several cases, for example, when the crop cut in a

young condition had actually less moisture than the same crop taken later in the season. Generally the water content decreased as the grass was getting old, and White clover, being less fibrous, generally contained more moisture than the other three species.

With respect to total nitrogen content in the individual species, the results show that it tended to fall as the plant reached and passed the flowering stage and then rose again for the aftermath. This is particularly noticeable when consideration is given to samples taken from the same locality at different times during the growing season and these results are common for all four species. The results for Cocksfoot and Rye-grass are, however, rather irregular. For example, samples 20, 24, 25 and 29, Cocksfoot and Rye-grass aftermath taken in August 1939, contained less nitrogen than their corresponding old aftermath samples taken in September and October 1939. This is probably due to the presence of some dead grass in the earlier samples. Where the aftermath had been grazed by sheep the rise in total nitrogen is even more pronounced due presumably to the fact that the grass had been kept short with many actively growing shoots.

As to the difference in nitrogen content among

the different species it will be noticed that White clover, as might be expected, invariably contained two or three times as much nitrogen as the grasses. There are a few exceptions to this general statement. For instance, Rye-grass which had been grazed by sheep and was sampled in October 1939 and again in September 1940 showed a high nitrogen content, 4.3 and 3.1 respectively; Cocksfoot, again grazed by sheep and sampled in September 1939, 3.98%; Timothy, grazed by sheep and sampled in September 1939 and June 1940, 2.8% and 3.1%. Generally speaking there are not striking differences between Cocksfoot and Rye-grass; the number of samples of Timothy is not so great and the figures are similar to those for the Cocksfoot and Rye-grass.

In considering the effects of manurial treatment on the total nitrogen content in grasses, it will be seen, as shown in Table VI, that the application of slag to Rye-grass produced an increase of 0.15% of nitrogen. But the reverse was the case with Cocksfoot where the plots receiving more slag produced a sample containing 0.07% less of nitrogen than that from plots received less slag. Lime affected the nitrogen content adversely both in Cocksfoot and Rye-grass. The results obtained from a later dressing of sulphate of ammonia

was not unexpected for it confirmed previous unpublished results obtained at Hoghall. The fertilizer has produced an increase of about 30% over the control. Increase in protein content of grass by nitrate of soda and sulphate of ammonia has also been reported by Moon (1939, 4). Moon also found that the application of superphosphate produced a significant increase and carbonate of lime had no influence on the nitrogen content. The beneficial effect of sulphate of ammonia is in agreement with the result in the present investigation. The effects of phosphate and lime, as stated by Moon, however, have not been confirmed.

With regard to the influence of season, since all the samples were collected during summer and autumn, the effect of season that can be shown was limited to to this short period. So far as this is concerned, season did exert some effect on the nitrogen content of grass. In general, the nitrogen content was higher in June, decreased gradually during July and August, and rose again in September when the aftermath was growing especially after being grazed. Samples 9, 24, and 33 of Cocksfoot, which were taken in June, August and September 1939, however, did not show the same variation. This is due to the fact that sample 9 was

fully ripe when cut, and the aftermath cut in September was old. The trend of the variation of nitrogen content during different seasons appears to be in accordance with the stage of growth, and the effects of these two factors seem to overlap each other.

The effect of locality was not significant. Since the nitrogen content varied greatly according to the stage of growth, which is dominant over other factors, and the samples taken from different farms were not exactly at the same stage of growth, it is impossible to compare the influence of different local conditions.

CAROTENE CONTENT OF FRESH GRASSES

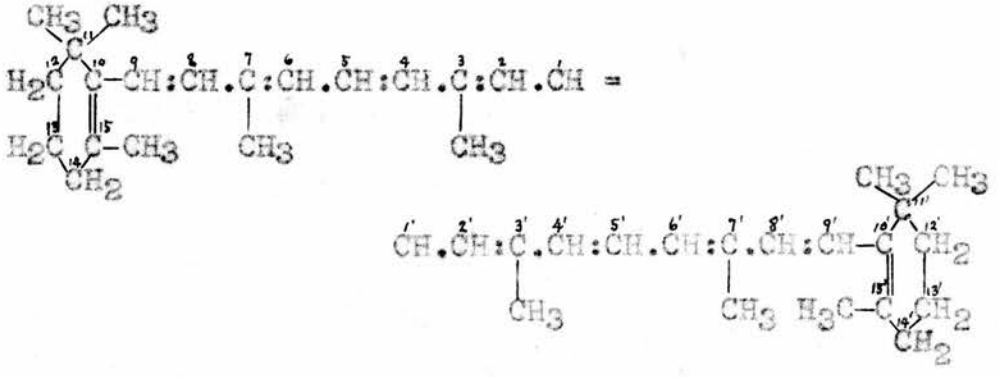
INTRODUCTION. Carotene is one of the carotenoids which are soluble in fat and found widely distributed in plants and animals. In the diet of herbivora, carotene and xanthophyll are the main carotenoids. Carotene ($C_{40}H_{56}$) is a hydrocarbon; xanthophyll ($C_{40}H_{56}O_2$), a mixture, lutein and zeaxanthin predominating, is an alcohol. The ratio of carotene to xanthophyll in fresh grass is fairly constant (1:2), but varies greatly in dried grass. Carotene was first obtained in crystalline form from carrots, and its chief source in nature is in association with chlorophyll in all green foliages. In its pure state, carotene forms reddish brown crystals, insoluble in water, sparingly soluble in alcohol and ether, and soluble in chloroform, benzine and petroleum ether.

The importance of carotene lies in the fact that it is a precursor of vitamin A. Animals have the ability of converting carotene and some other carotenoids (kryptoxanthin, myxoxantin and aphanin) into vitamin A, and are primarily dependent on carotene for their supply of vitamin A. Although birds accumulate xanthophyll in their blood and body fat to the exclusion of carotene, most of the

mammals appear to absorb carotene preferentially to xanthophyll. The site of the conversion of carotene into vitamin A is generally held to be the liver, under the influence of a thermolabile enzyme, provisionally designated carotenase.

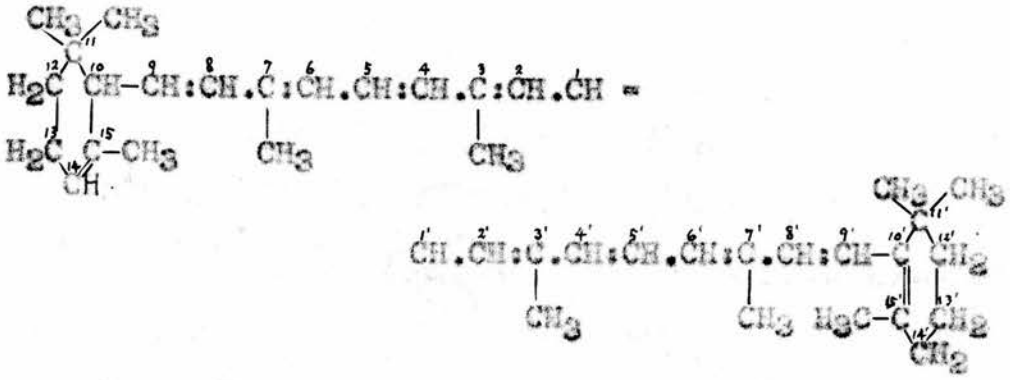
The absence of both vitamin A and carotene from the diet of animals gives rise to an eye infection, called xerophthalmia, and consequently blindness, and this is followed by loss of weight, degeneration of nervous system, and an all-round lowering of the power of resistance of the body against diseases.

CHEMISTRY. Carotene exists in three isomeric forms, designated α, β, and γ. β-carotene, the principle constituent of most leaf carotenes, melting point 184°, optically inactive, has the following constitution:

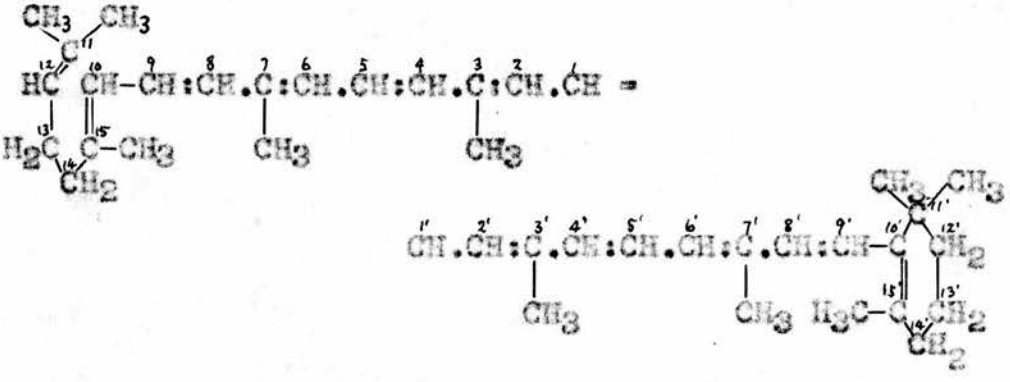


α-carotene, melting point 187°, dextrorotatory, has a double bond in the ring between C^{14} and C^{15} instead of between C^{10} and C^{15} and the double bond between

$C^{10'}$ and $C^{15'}$ remains unaltered.



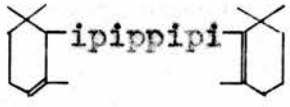
γ-carotene, melting point 178°, optically inactive, has a double bond between C^{11} and C^{12} in addition to that between C^{10} and C^{15} and the ring cleaves between C^{10} and C^{11}.



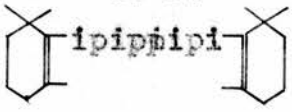
The central polyene chain from C^{8} to $C^{8'}$ is made up of four isoprene units arranged in pairs which are united in reversible order at C^{1} and $C^{1'}$ and may be abbreviated as ipippipi, ip denoting an isoprene unit. The terminal groups consist of two β-ionone rings at both ends in β-carotene; one β-ionone ring and one α-ionone ring in α-carotene; and one β-ionone

ring and one broken ring in γ-carotene. The polyene chain is common to a large number of carotenoids, but the terminal groups may consist of substituted or unsubstituted rings. The carotenoids which can act as precursors of vitamin A are those that possess intact one half of the β-carotene molecule. They include kryptoxanthin (Morton, 1940), myxoxanthin (Heilbron and Lythgoe, 1936; and Morton, 1940) and aphanin (Tischer, 1937) in addition to the three isomeric carotenes, and their formulas may be expressed as:

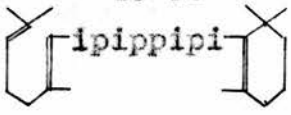
α-carotene ($C_{40}H_{56}$)



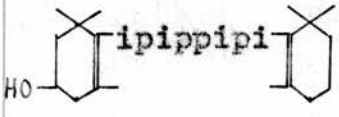
β-carotene ($C_{40}H_{56}$)



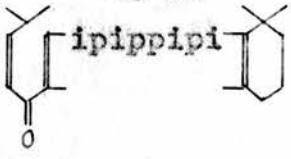
γ-carotene ($C_{40}H_{56}$)



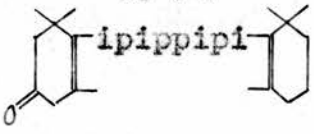
Kryptoxanthin ($C_{40}H_{56}O$)



Myxoxanthin ($C_{40}H_{54}O$)

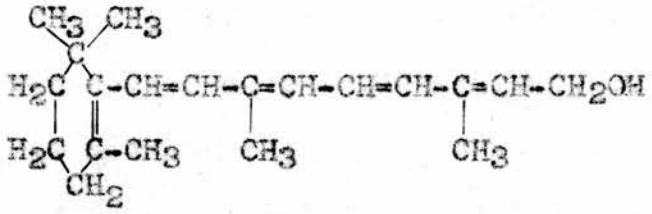


Aphanin ($C_{40}H_{54}O$)



Between the three carotenes and vitamin A, there are interesting connections. Karrer, Morf, and Schöpp (1931) made an investigation of highly purified vitamin A and suggested the following formula for it which is closely related to half of the β-carotene

molecule.



Although the mechanism of the conversion is still unknown, it has been commonly postulated that one molecule of β-carotene, having a symmetrical formula, produces two molecules of vitamin A by the addition of two molecules of water, and that the fission occurs at the C^{1}-$C^{1'}$ double bond. The equation can be shown as follow:

(1) $C_{40}H_{56} \xrightarrow{2H_2O} 2C_{20}H_{29}OH$

536 — 572 parts by weight

1 g. — 1.067 g.

While α-carotene and γ-carotene, being unsymmetrical, are only half as efficient as β-carotene, producing one molecule of vitamin A with some decomposition products.

(2) $C_{40}H_{56} \longrightarrow C_{20}H_{29}OH$ + decomposition products.

UNIT OF VITAMIN A AND ANIMAL REQUIREMENT. Since carotene is the precursor of vitamin A, it is advisable to deal with vitamin A in regard to its unit and animal requirement. The I.U., denoting the International Unit, of vitamin A was adopted at the

International Vitamin Conference held in 1931 and maintained at the 1934 Conference. One I.U. is defined as the vitamin A activity of 0.6 microgram (0.6γ) of pure β-carotene of the International Standard Preparation. The potency of pure β-carotene is therefore 1.66×10^6 I.U./g., so that the potency of vitamin A is 1.56×10^6 I.U./g. ($\frac{1.66 \times 10^6}{1.067}$, see equation 1 on page 38), where as all provitamins other than β-carotene have a potency of about 0.83×10^6 I.U./g. But according to bioassays carried out with rats, it has been shown that vitamin A is, weight for weight, twice as potency as β-carptene and thus vitamin A has a potency near 3.0 to 3.3×10^6 I.U./g. Vitamin A is estimated spectroscopically by utilizing the absorption maximum at 325 mμ and the conversion factor generally accepted is ; $E^{1\%}_{1cm}325m\mu$, l=1,600 I.U./g.

For the detection and estimation of vitamins, biological methods have also been used. They consist of feeding a ration free from vitamin A but complete in other respects, until the supply of vitamin A stored in the animal is exhausted, and then feeding weighed quantities of the feed to be tested in conjunction with the ration free from vitamin A. The amount of supplemental food which produces an average

increase in weight of 3 g. a week for 8 weeks following depletion of the body store of vitamin A, is said to contain one unit of vitamin A. Since most of the biological tests are carried out with rats, it is designated as "rat unit". Fraps and Treichler (1933) stated that it has been found in the laboratory that the rat unit and I.U. are practically the same.

The animal body in health contains only small quantities of carotene, and is not equiped to assimilate large doses. The responses produced by β-carotene and vitamin A are very similar at minimum doses. At higher levels, however, the efficiency of vitamin A exceeds that of carotene. In all cases the vitamin A reserve of the newborn animal is low, and the rate of storage is much slower during the early period of rapid growth than when the growth-rate decreases later. Maximum storage is found in old animals. The requirements of birds and mammals are about 10 μg. of vitamin A per kg. body weight per day (Morton, 1940). In order to prevent the first symptom of night blindness, Guilbert, Miller, and Hughes (1937) found that a daily dose of 25-30 μg. of β-carotene or 6-8 μg. of vitamin A per kg. body weight is required by cattle, sheep, pigs, rats and horses. Sherwood and Fraps

(1932) found that White Leghorn pullets require 32 units per pound body weight per day for maintenance while laying and 6.3 units for each unit of vitamin A in the eggs. Milk cows, like chickens, require a large quantity of vitamin A for maintenance. Dann (1936) has shown that colostrum may contain 10-100 times as much as vitamin A as the later milk to enable the newborn to build up a reserve. The relative proportions of carotene and vitamin A in milk vary with the breed. Holstein and Ayrshire cows yield milk with little carotene but more vitamin A, whereas Guernseys give a cream more deeply coloured by carotene but less rich in vitamin A.

METHODS OF ESTIMATING CAROTENE. (a) PREVIOUS WORK.- Although β-carotene is practically the only carotene present in grass as stated on page 11, it has been the usual practice to include α-, β-, and γ-carotene as one substance under the name of "carotene". Willstätter and Stoll (1913) were probably the first to devise a method for determining carotene by extracting the tissue with acetone, saponifying the chlorophyll and removing it in the aqueous layer, and partitioning the xanthophyll and carotene between methanol and petroleum ether.

Most of the methods used for estimating carotene consist of two steps: the extraction of pigments and the estimation of carotene.

(1) Extraction of pigments. The processes of extraction can be divided into two groups. In the first group, the material is first ground in a mortar with sand or in a small mill, the pigments are then extracted with some solvent, and subsequently the chlorophyll is removed and the xanthophyll esters are split by saponification. In the second group, the original material is first saponified by heating with alkali to break up the tissue and then treated with solvents. Of the solvents employed for extraction, petroleum ether is the most commonly used. Saponification is generally carried out with alcoholic or aqueous potash.

Coward (1924) first tried to saponify the material before extraction with a solvent, and suggested that each step of the process should be carried out in nitrogen. Guilbert (1934) devised the process of boiling the sample for half an hour with a saturated alcoholic solution of KOH. The carotenoids are extracted with ethyl ether and the chlorophyllins and flavones are separated by washing with water. The

ether tract is distilled, and the residue is redissolved in petroleum ether from which the xanthophyll is removed with 90% methyl alcohol. Miller (1935, 2) extracted carotene by macerating the sample with acetone and sand before saponification. The method used by Ferguson and Bishop (1936) resembles closely that of Guilbert, but they preferred the use of 20% aqueous KOH to an alcoholic KOH, and the boiling lasts for two hours. They first determined the total carotenoids in the ether extract. After the evaporation of ether, the residue is redissolved in petroleum ether but the xanthophyll is removed with 92% methyl alcohol. The carotene left in the petroleum ether and the xanthophyll in methyl alcohol are determined separately, and their ratio is calculated. From this ratio the carotene of the original total carotenoid extract is calculated. A more rapid method has been used by Pyke (1936). The grass is finely ground and shaken in a centrifuge tube with a mixture of ether and KOH solution in methyl alcohol. After centrifuging, the ethereal layer is separated and evaporated. The pigments are dissolved in petroleum spirit and this is followed by the usual treatment with methyl alcohol. All these methods involve a preliminary extraction of

total carotenoids and the removal of the carotenoid solvent, followed by a partition between petroleum ether and methyl alcohol. The evaporation of solvent not only takes time but may also cause some loss of carotene. Moreover the carotene and xanthophyll are not of equal colour intensity (Willstätter and Stoll, 1913), and the determination of carotene by the ratio of the two carotenoids may lead to an overestimation of carotene.

Peterson, Hughes and Freeman (1937) modified Guilbert's method by omitting the use of ethyl ether and extracting the saponified material directly with petroleum ether. They obtained similar results to those obtained by using ethyl ether. Moon (1939, 2) made a similar modification. The essential difference is that the sample is saponified by boiling the sample for 1½ hours with aqueous KOH, followed by filtration and extraction of the grass residue with alcohol. In this way, a complete breakdown of cellulose, which is essential for efficient extraction, can be attained; and the undesirable precipitate, which hinders subsequent extraction and is produced by the use of hot alcoholic potash, can be avoided. The results obtained are reproducible with considerable

accuracy.

The petroleum ether solution of carotene thus obtained, however, is not pure carotene and contains some other coloring matters. The impurities can either be estimated by spectrophotometric methods; or separated by chromatographic or selective absorption methods by passing the solution through a well-packed column of suitable absorbents such as alumina, lime, calcium hydroxide, calcium carbonate, and magnesia. By using spectrophotometric methods, Gillam (1933) found that the absorption data in chloroform were as follows: $E^{1\%}_{1cm}$ (463mμ) = 1900 for carotene; $E^{1\%}_{1cm}$ (455mμ) = 1570 for xanthophyll. Ferguson (1935) obtained 2200 for carotene in chloroform at 463mμ. In 1935, Gillam obtained 2500 in petroleum ether and 2200 in chloroform for β-carotene, both at 450mμ. Recently, the E value of β-carotene obtained by Seaber (1940) was 2520 at 450mμ in petroleum ether and agreed closely with that obtained by Gillam. By the chromatographic methods, Fraps and Kemmerer (1939) found that the percentage of impurities in the petroleum ether solution extracted from alfalfa ranges from 0 to 23% using magnesium hydroxide as absorbent. They designated

the carotene estimated in the original solution as "crude carotene" and that after being purified as "pure carotene". Seaber (1940) use Merk's alumina in 3% acetone and found that the ratio between the pure carotene and the crude carotene in dried grass and alfalfa varied from 0.7 to 0.8.

For general purpose, however, it has been the usual practice to determine the colour value in terms of carotene in the original petroleum ether solution without further analysis, in spite of the fact that the pure carotene is sometimes only 80% of the figures obtained.

(2) Estimation of carotene. The carotene content in the petroleum ether solution is estimated by comparison with a pure carotene solution or more commonly with some other standard solutions, which can be compared against a pure carotene solution and the values so obtained can be used for all subsequent determinations. Kuhn and Brockmann (1932) used an azobenzene solution of 14.5 mg. in 100 ml. of 95% alcohol which is equivalent to one containing 0.285 mg. carotene per 100 ml. in light petroleum. Ferguson (1935), and Fraps and Kemmerer (1937) used a 0.1% solution of potash dichromate, which is equivalent

to one containing about 0.61-0.63 mg. carotene per 100 ml. in petroleum ether. Ferguson (1935) has also constructed a curve for Lovibond readings plotted against carotene. According to his reading, a carotene solution containing 0.1015 mg. per 100 ml. corresponds to 1.6 yellow units.

The instruments in common use for estimating carotene are the colorimeter, tintometer and photo-electric absorptiometer. Nessler tubes can also be used for direct comparison, but it is difficult to get consistent results by this method.

(b) METHOD ADOPTED AND DETAILS OF TECHNIQUE.-

(1) Method adopted and instrument used. In this investigation, the procedure for extracting carotene was based on the method devised by Moon (1938, 3). This method involves a direct extraction of carotene immediately after the saponification of chlorophyll and without a preliminary determination of total carotenoids, and is suitable for rapid routine work and the analysis of several samples simultaneously. For the colorimetric estimation of carotene, a Lovibond Tintometer has been used.

(2) Details of technique. Five g. of finely chopped fresh grass were weighed out from the bulk

sample and transferred to a conical flask. Forty ml. of 20% KOH were added and the mixture was boiled for 1½ hours under a reflux condenser. During boiling, it was advisable to rotate the flask to keep the sample from collecting on the sides. After boiling, the contents of the flask were cooled and filtered through small Büchner funnel under reduced pressure. The residue was transferred to a beaker and extracted four times with 25 ml. portions of ethyl alcohol and once with petroleum ether until the extraction was colour-free. After each extraction, the mixture was poured through the same filter. The grass residue remained coloured green due to the presence of potassium isochlorophyllin. In the mixed extracts, the reaction between alkali and alcohol produced some precipitate which should be filtered under reduced pressure and washed with petroleum ether.

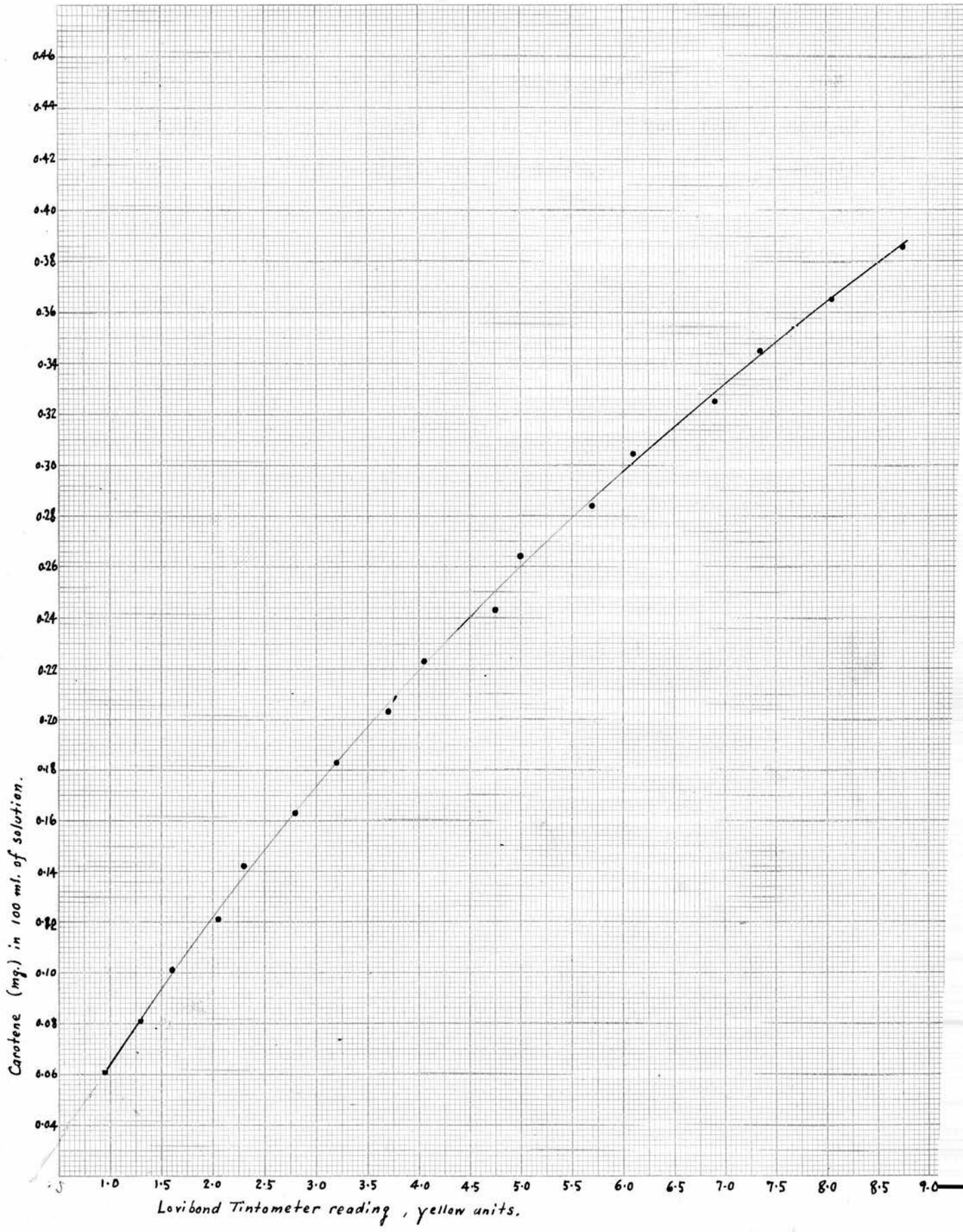
The combined alk. alcohol and petroleum ether extract was then transferred to a 500ml. separating funnel and well shaken. After settling, the alkaline alcohol solution containing most of the chlorophyllines and flavines was drawn off from the bottom of the funnel and re-extracted three or four times more by shaking with fresh petroleum ether until the yellow colour

in the final petroleum ether extract was entirely removed by 92% methyl alcohol indicating that the extraction of carotene is complete. In case any colour remained on testing with 92% methyl alcohol, further extraction of the original alcohol solution with petroleum ether was necessary. In carrying on the extraction it is more convenient to use two funnels alternatively.

The total petroleum ether solution. containing both carotene and xanthophyll, was transferred to a separating funnel and extracted exhaustively with 92% methyl alcohol, 3 or 4 extractions usually being sufficient to remove all the xanthophyll. The petroleum ether, now, free from xanthophyll, was then washed with distilled water to get rid of any alkali left. The resulting solution of carotene in petroleum ether was quite clear and the volume was measured in a cylinder. It was ready for colour matching.

The colour matching was carried out by the use of a Lovibond Tintometer, using a "daylight" lamp. It consists of a series of colour slides. The carotene solution was placed in a 1-cm. glass cell and the colour matching was done by moving the yellow glass slides until the equivalent density of colour was obtained. To get a close match, it was sometimes

Fig. I. Curve (Ferguson, 1935) for converting Lovibond Tintome
reading into mg. of carotene per 100 ml. of solution.



necessary to use red slides as well, but since the red reading was usually very small, only the yellow units were considered. As the colour of the petroleum ether solution was usually too dense to match directly, appropriate dilutions were made. In order to get accurate readings, it is preferable to read at a point not higher than 4 to 5 yellow units, and an average of three independent readings is taken. By use of the curve on the opposite page, which was plotted from Ferguson's data and curve (1935), the Tintometer reading was converted into concentration of carotene and expressed as "parts per million" of the dry matter.

(3) Example of calculation of results. The method of calculation of results can be explained by the following example:

Weight of fresh sample	5 g.
% of dry matter, say	20%
Volume of petroleum ether solution, say	50 ml.
Tintometer reading, say	3.0, which is equivalent to 0.1726 mg. of carotene per 100 ml. of solution.

$$\frac{0.1726 \times 50/100}{5 \times 20/100} \times 1{,}000 = 86.3 \text{ parts per million.}$$

This can be shortened into the following formula:

$$\frac{C \times V}{W \times D} \times 1{,}000 = \text{p.p.m.}$$

C is mg. of carotene per 100 ml. of solution (from the curve opposite page 80); W is weight of grass sample; V is volume of petroleum ether solution in ml.; D is percentage of dry matter; and p.p.m. is parts per million.

(4) Preliminary test of the conversion factors. Since the Tintometer reading varies with different observers, a preliminary test of the conversion factor was made, with several carotene solutions extracted from grass, by matching the colour with the Tintometer first and checking in a Klett Colorimeter against 0.1% potassium dichromate solution. The carotene solution was placed in the left cup of the colorimeter and set at 20 mm. on the scale, and the matching was carried out by varying the height of the potassium dichromate column in the right cup. The results are shown in Table VII.

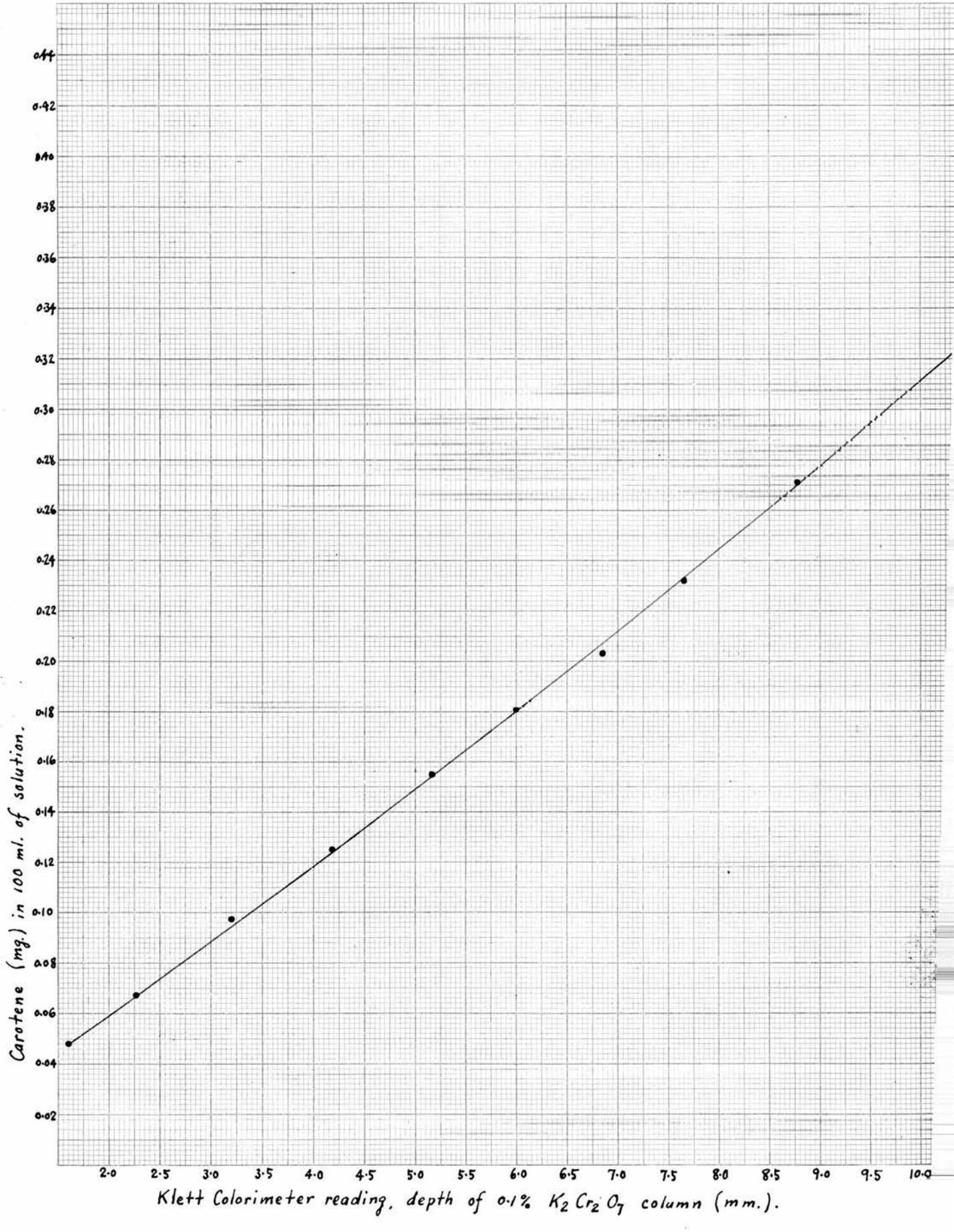
Table VII.

Lovibond Tintometer reading		Klett Colorimeter reading	
Yellow units	Carotene per 100ml. solution (mg.)	Depth of 0.1% $K_2Cr_2O_7$ column (mm.)	Carotene per 100ml. sol. (mg.)
1.2	0.075	2.5	0.074
1.3	0.081	2.7	0.080
1.7	0.106	3.6	0.108

Table VIII. Summary of carotene-contents (p.p.m.)

Stems and leaves				Heads or flowers			
W.C.	C.	R.	T.	W.C.	C.	R.	T.
456.3							
182.5				94.6			
142.5				72.4			
325.7							
552.3		60.0					
252.3	91.8	4.3			60.4	20.0	
		137.3					
303.9	138.2	260.9					
487.7		60.4				79.5	
		113.8					
		188.6					
322.5	57.3	53.4		118.7	90.0	73.5	
194.3	147.6	95.5					
	102.0	138.0					
508.6							
	146.1						
	42.4				38.0		
	385.0						
	140.5						
	257.9						
	122.6						
	225.4						
	300.0						
	381.8						
	165.7				110.8		
	211.1				148.3		
		87.5					
		27.1				74.9	
		254.0					
354.8		135.3				151.1	
453.9		225.3					
		82.4				108.2	
			194.2				
			111.7				97.7
			275.3				
			246.7				
			207.7				124.8
			222.2				
			223.1				
			171.4				

Fig. II. Curve (Ferguson, 1935) for converting Klett Colorimeter reading into mg. of carotene per 100 ml. of solution.



2.4	0.144	4.9	0.146
2.5	0.149	5.0	0.149
2.7	0.159	5.2	0.156
2.9	0.170	5.7	0.170
3.0	0.173	5.8	0.173

X These figures were calculated from the curve on the opposite page which was plotted from Ferguson's data (1925).

As the above table shows, the figures obtained with the Tintometer agreed quite well with those obtained with the Klett Colorimeter.

RESULTS AND DISCUSSION. The carotene-content of the samples are recorded in Tables VIII and IX. The particulars of each sample are contained in the tables opposite page 29.

A study of the carotene-contents among the four species will reveal that White clovers were superior to the other three species. The highest figure obtained for carotene was about 552 p.p.m. for a sample of very young White clover, the lowest figure for clover was 142 p.p.m. for a sample of old material taken in August. The figures for the grasses were generally much lower and varied in the case of Cocksfoot from 42 p.p.m. for a sample fully ripe taken in July to 385 p.p.m. for a sample of aftermath taken

in September which had been grazed by sheep. For Rye-grass, the figures with one exception varied from 27 p.p.m. for a sample of ripe material taken in July to 261 p.p.m. for a sample taken in September after grazing. There is one value as low as 4 p.p.m. for very old material taken in August; most of the seeds had been shed. In the case of Timothy the values ran from about 112 p.p.m. for a ripe sample taken in July to 275 p.p.m. for a young aftermath which had been grazed by sheep and was sampled in September.

With respect to the effect of the stage of growth, it will be seen that all four species showed a similar trend of variation. The carotene-contents were greatest when the plant was young, tended to decline at flowering or heading, decreased rapidly with fading or ripening, and rose again for the aftermath. This general statement holds true especially in White clover and Timothy, and it appears that the carotene-contents of White clover and Timothy were less affected by age than those of Rye-grass and Cocksfoot. The figures for White clover range from 142 p.p.m. for a sample which was fading to 552 p.p.m. for a very young sample, and those for Timothy range from 112 p.p.m. for a ripe sample to 275 p,p.m. for a young

Table IX. Summary of carotene-contents (p.p.m.) of grass grown under different manurial treatments.

Stems and leaves	Heads or flowers
Cocksfoot	
111.1	89.7
135.2	96.9
Rye-grass	
73.8	84.5
76.4	95.2
Cocksfoot	
112.9	92.1
125.6	91.8
Rye-grass	
77.2	81.4
77.3	90.0
Rye-grass	
64.2	88.3
99.2	103.0

aftermath after grazing. The results for Cocksfoot and Rye-grass had also a wide range of variation and were rather irregular. For example, some earlier aftermath samples (NO. 20, 24, 25, and 29) contained less carotene than the corresponding later samples (No. 26, 33, 34, and 39) from the same locality. This was no doubt due to the fact that the earlier samples were not entirely free from old dead grasses left after cutting. Moon (1939, 1) carried out some investigations on the composition of grass in 1938 and found that the carotene-contents in a mixed herbage showed a marked fall during April and May 1938 due to drought, and remained constant until flowering began, when a marked loss occurred. The exceptional decline of carotene in the early season of Moon's experiment was undoubtedly affected by the unusual weather. In the present investigation, no samples were collected in April, but the general variation of carotene-contents seems to be more or less along the same lines.

The results shown in Table IX indicate that the effect of ammonium sulphate was very remarkable, producing an increase of 25-35% over the control, which is in good agreement with the results got by previous workers (Virtanen et al., 1939; Ijdo, 1936;

Thomas and Moon, 1938; and Moon, 1939, 4; see page 13). As to the effects of slag and lime on carotene-content, the results reported by other workers are variable (see page 13). But in this investigation, both slag and lime caused some increase of carotene in the leaves and stems, and in the heads or flowers with the exception of the carotene-contents in the heads of samples 52 and 53. Their effects were, however, not very striking. This might be ascribed to the fact that the control plots, which received no fertilizers, did not produce grasses well enough to justify sampling, and the samples taken for comparison were from plots which also received some fertilizer, though less in quantity.

The range of carotene-contents during different seasons are summarized in Table X.

Table X.

Month	W.C. (p.p.m.)	C. (p.p.m.)	R. (p.p.m.)	T. (p.p.m.)
May	- - -	166-382	- - -	- - -
June	355-552	123-211	60-135	194-247
July	143-323	42-141	27- 53	112-208
August	194-509	92-225	4-137	171
September	304-454	138-385	189-261	222-275
October	- - -	102	138-254	- - -

The results show that the carotene-contents of all four species were high in May and June, fell in July and August, and rose again in September and October.

In a number of cases where the grass had reached the flowering stage the heads were examined separately from the stems and leaves for carotene-contents. Generally speaking, the amount of carotene in the heads of flowers was less than in the stems and leaves. But a few instances occurred in which the reverse was the case, for example, a sample of Cocksfoot at flowering stage taken in July showed only 57 p.p.m. of carotene from the stems and leaves and 90 p.p.m. in the heads and in the case of Rye-grass all samples which were examined in this way showed more carotene in the heads than in the stems and leaves: these Rye-grass samples were taken in different places in June, July and August.

One of the most striking features of the results is the very close parallelism between the nitrogen and carotene figures. This correlation is summarized in Table XI and is best shown in the attached graphs where nitrogen and carotene have been plotted for each species and where all the nitrogen results have been plotted against the corresponding carotene

Fig. III. Correlation between total N and carotene of White clover during different stages of growth.

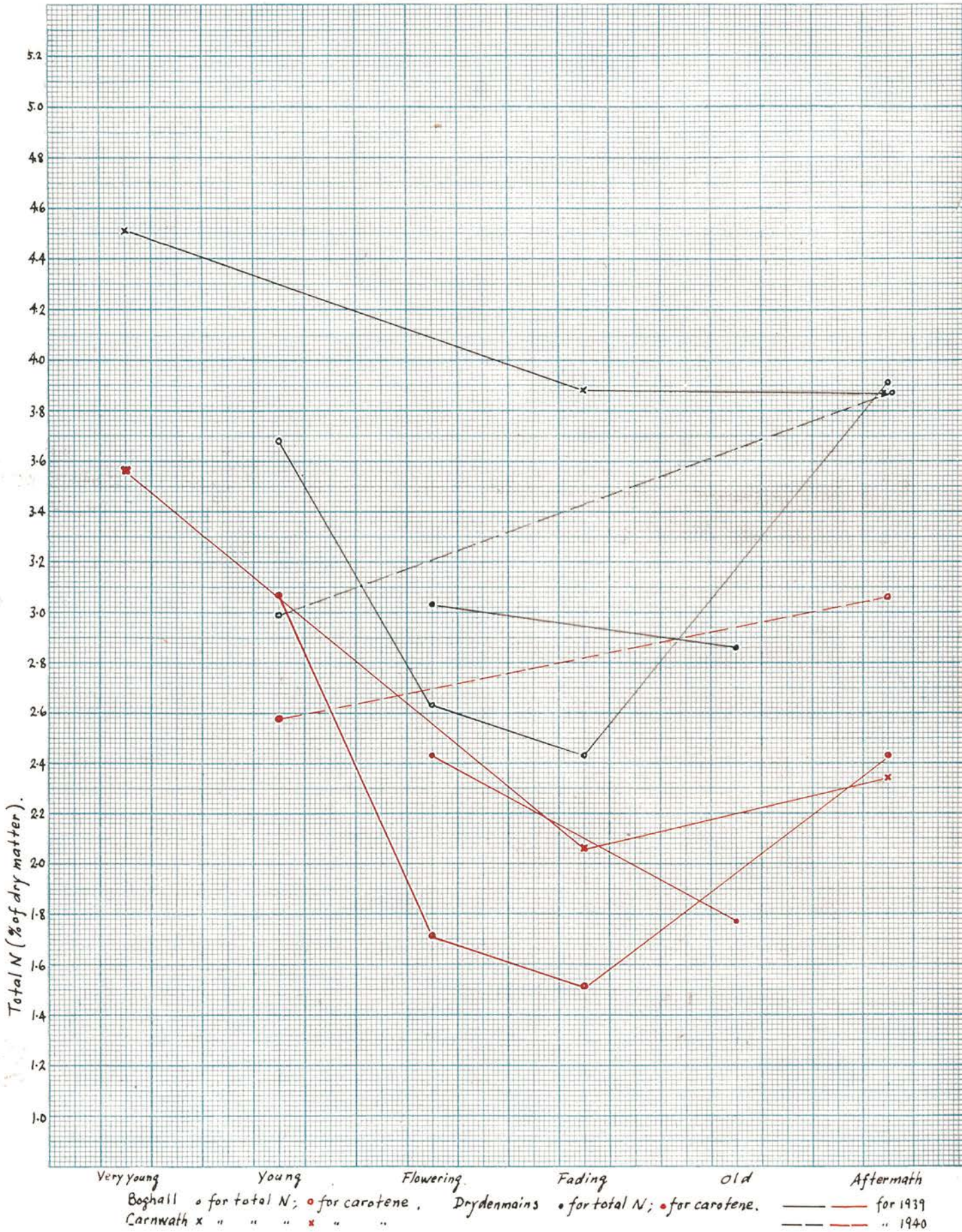


Fig. IV. Correlation between total N and carotene of Cocksfoot during different stages of growth.

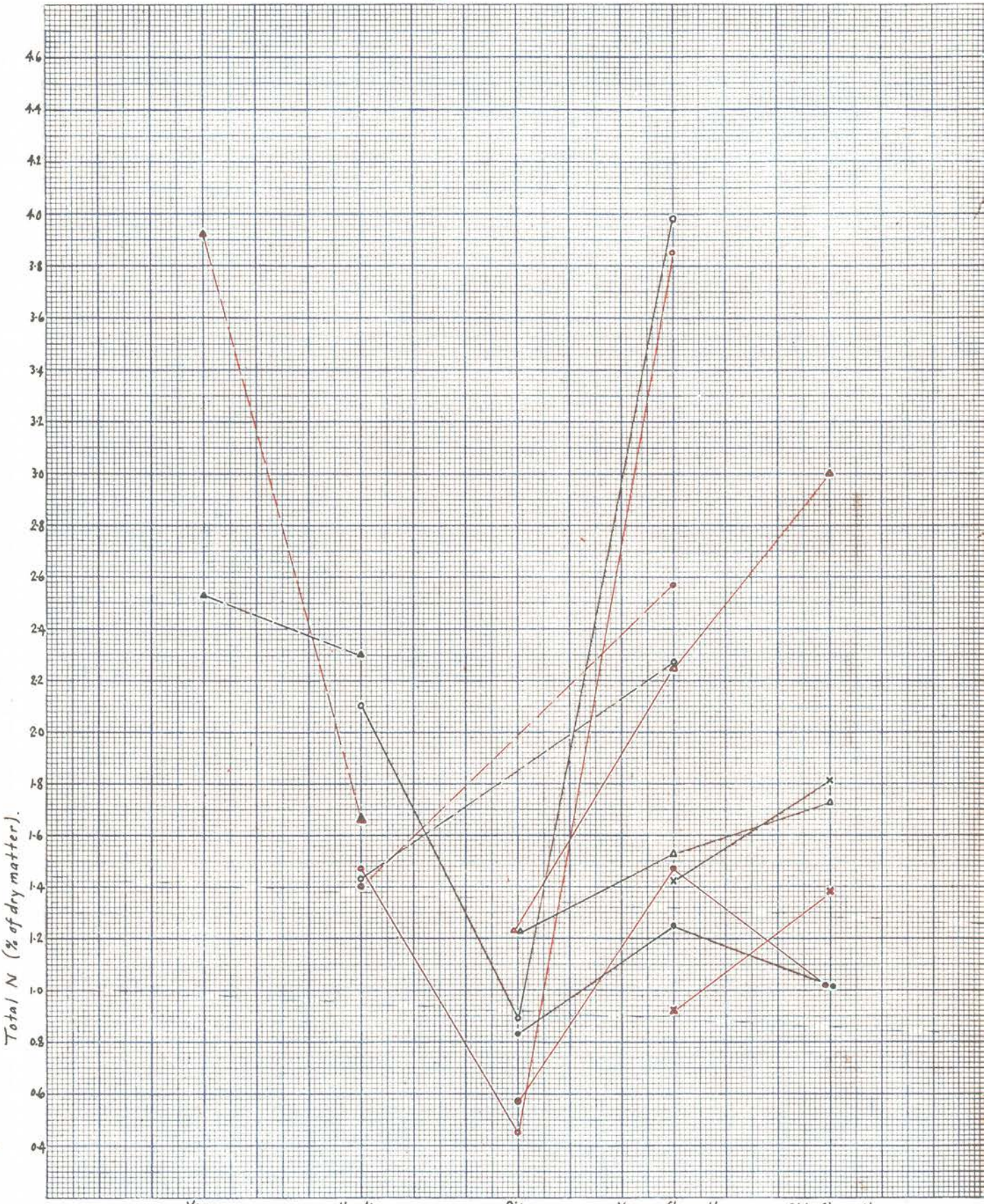


Fig. V. Correlation between total N and carotene of Rye-grass during different stages of growth.

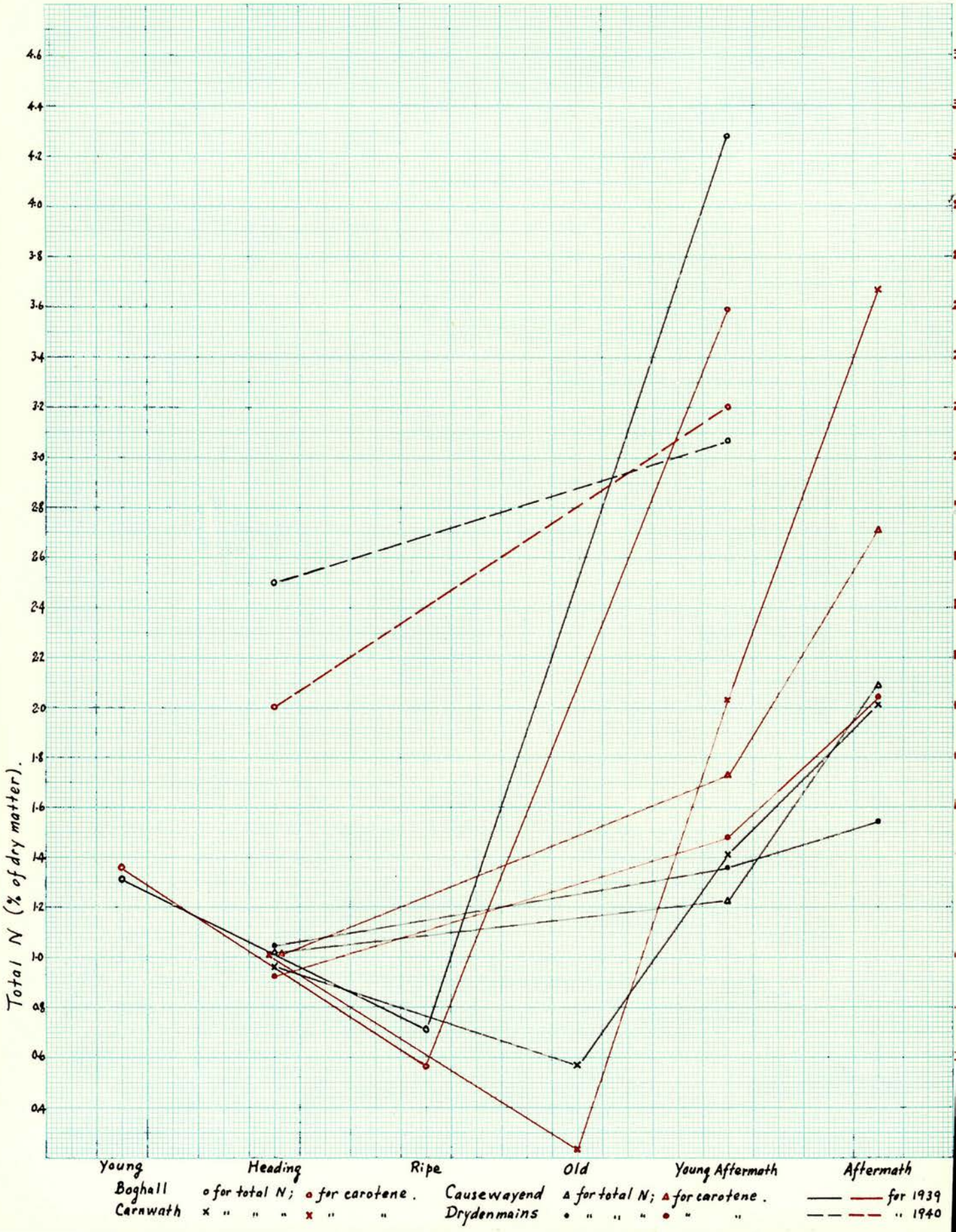


Fig. VI. Correlation between total N and carotene of Timothy during different stages of growth.

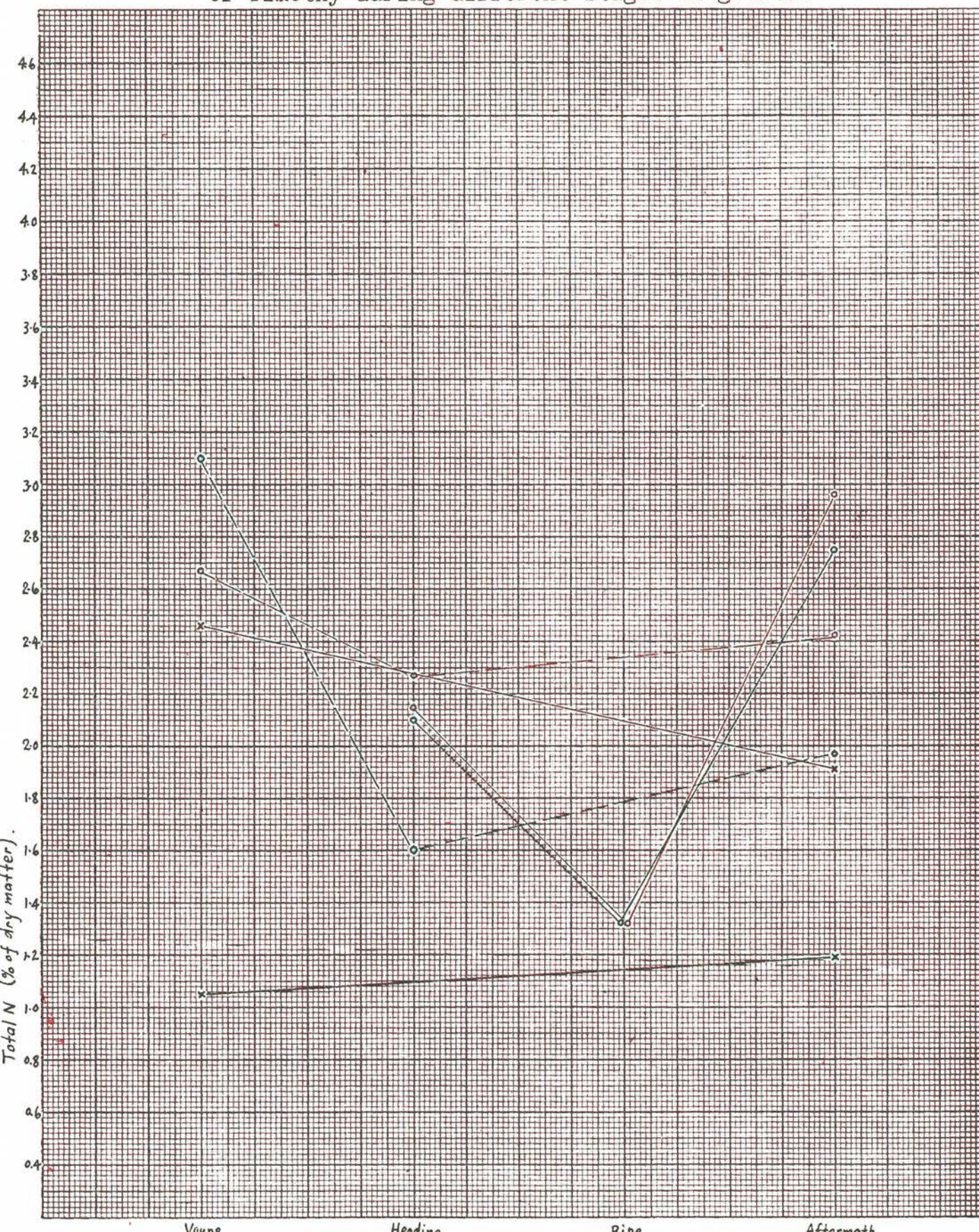
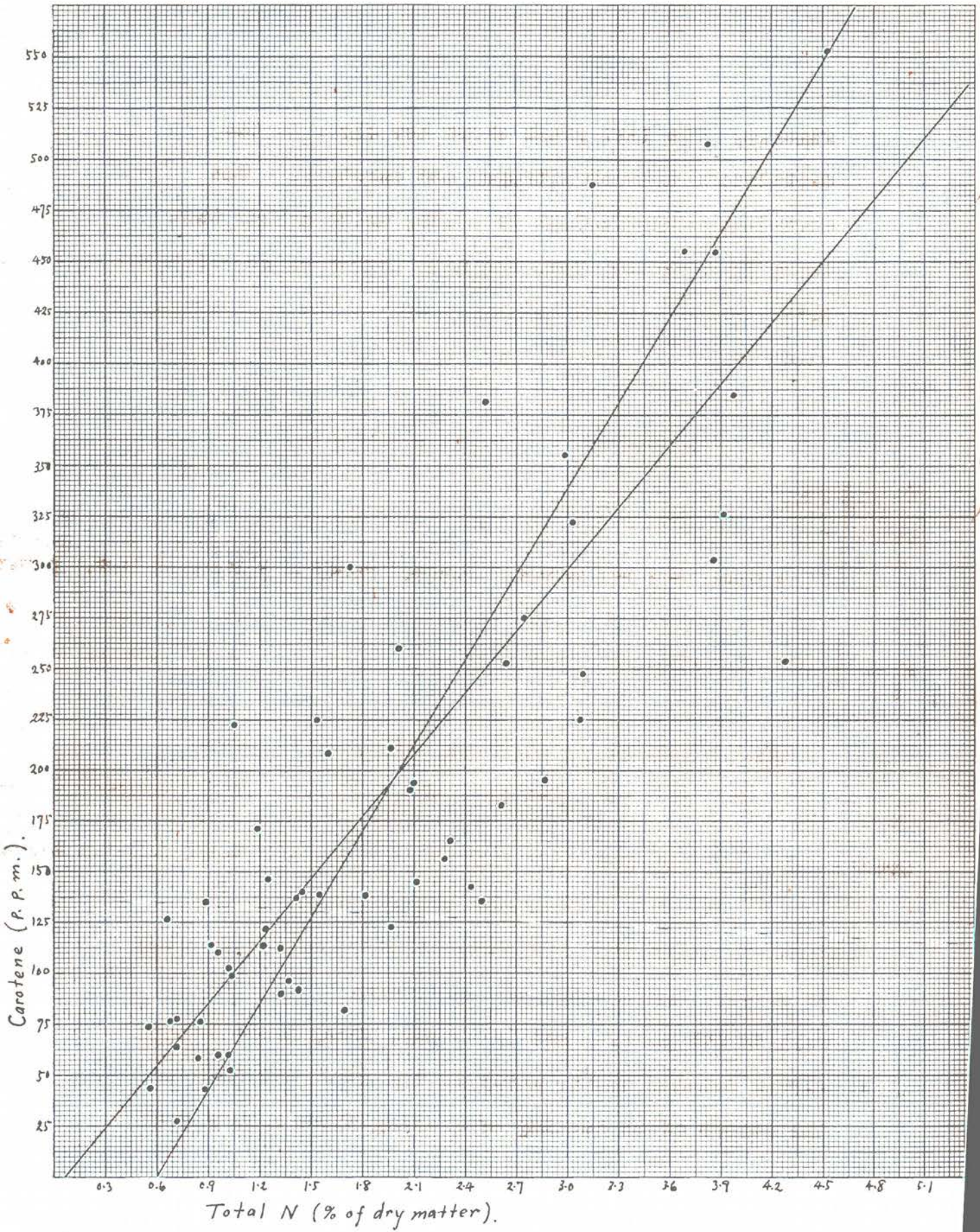


Fig. VII. Correlation between total N and carotene.



figures. The last graph shows how close is the correlation between nitrogen and carotene. The correlation coefficient is +0.85, which meands that there is no doubt about the high degree of association between carotene and total nitrogen. The regression lines in Fig. VII were drawn according to the following equations:

$$C = 102N - 9; \qquad N = 0.0071C + 0.6$$

Table XI.

Stage of growth	W.C.		C.		R.		T.	
	N % D.M.	Carotene p.p.m.	N % D.M.	Carotene p.p.m.	N % D.M.	Carotene p.p.m.	N % D.M.	Carotene p.p.m.
Young	3.7-4.5	456-552	2.5	382	1.3	88	1.1	228
Flowering or heading	2.6-3.2	183-488	1.4-2.3	140-211	1.0-1.7	53- 82	1.6-2.1	194-208
Old	2.4-2.9	143-252	0.8-1.2	42- 57	0.6-0.7	4- 27	1.3	112
Aftermath	3.9-3.9	304-326	1.0-1.8	92-300	1.2-2.1	96-189	1.1-2.0	171-222
Grazed	3.0-3.9	355-454	2.3-410	258-385	2.0-4.3	135-261	2.8-3.1	247-275

This close correlation between nitrogen and carotene means that the observation on locality, stage of growth, and species, for nitrogen, apply equally well to carotene. That is to say, young grass or clover sampled either early in the season or late in the season after cutting or grazing shows a very much

higher content of carotene than the same crop which has reached the flowering stage or has become old and fibrous.

The relationship between carotene and protein has been dealt with by Moon who showed that the correlation between carotene-contents and both the true and crude protein was highly significant and positive (1939, 1). He also reported (1939, 4) that the carotene-content was closely associated with the protein-content under manurial treatments which produced little or no effect on these two constituents, but the correlation was not so close when the manurial treatments produced increases in carotene or protein. The results obtained in the present investigation, however, indicate that the beneficial effect of the application of ammonium sulphate on the carotene-content and on the total nitrogen seems to be about the same, and these two constituents are very closely associated.

One more interesting point is the beneficial effect of grazing. This applies to all four species, at any stage of growth, and to both nitrogen- and carotene-contents. As Table XI shows the nitrogen contents in the grazed White clover samples were much higher than the samples at heading or flowering and

about the same as aftermath, and their carotene-contents were even higher than aftermath and only slightly lower than young samples. The effect of grazing is more significant in the grasses. Both the nitrogen- and carotene-contents in grazed samples of all the three grasses were markedly higher than the young samples collected in May and June.

LEAF PROTEIN AND METHOD OF EXTRACTION

THE STRUCTURE OF LEAF CELL AND THE PROPERTIES OF LEAF PROTEINS. In studying the leaf proteins and their extraction it is desirable to give a brief accoumt on the structure of the leaf cell. An individual leaf cell is enclosed in a cellulose wall and consists of a peripheral layer of protoplasm surrounding a large vacuole or sap cavity. The ground mass of the protoplasm is the cytoplasm which is a more or less transparent viscid liquid embedding the nucleus, chloroplasts and other inclusions. The cytoplasm and its attendant organs are emulsion structures of protein, lipoid, pentosan, etc.; while the vacuolar sap is a solution of inorganic salts, sugar, amino acids and other products of relatively low molecular weight. After grinding, some of the chloroplasts and nuclear material are dispersed into colloidal solution, and the cytoplasmic protein is dispersed into a true solution virtually.

The properties of the leaf proteins vary according to source. Generally, the cytoplasmic proteins, which represent the protein fraction of the cytoplasm, are insoluble in water but soluble in slight excess of either of acid or alkali. When

hydrated, their solubility is at a minimum between the limits of pH 4.0 and pH 5.0. Below pH 4.0, they are soluble slowly and their solubility increases with decrease of pH to 2.5, beyond which they precipitate again. Above pH 5.0, they are readily and increasingly soluble with increase of pH and can be precipitated by $(NH_4)_2SO_4$ and other salts. They are denatured by treating with alcohol and ether. The protoplasmic proteins, which include the cytoplasmic proteins and chloroplastic material, exhibit the same solubility as the cytoplasmic protein, flocculating within the limits of pH 4.0 to 5.0, freely soluble in alkali and slowly in acid.

PREVIOUS WORK ON THE EXTRACTION OF LEAF PROTEINS.

Rouelle (1773) was probably the first worker to prepare protein from leaves by fractional heat flocculation. Since then, no serious attempts at preparing leaf protein were made until 1919, when Osborne and Wakeman, and Chibnall and Schryver, almost simultaneously, prepared the proteins from leaves for analysis. Osborne and Wakeman (1920) worked with spinach, grinding fresh spinach leaves with water, centrifuging the mash, and adding 20% of alcohol to the green colloidal solution to precipitate the protein. Chibnall and

Schryver (1920) worked with cabbage, grinding the leaves with ether-water to act as a cytolytic agent, pressing out the resulting mash through muslin and heating the green colloidal liquid to 80°C to coagulate the protein. The protein extractions prepared by simple grinding with water or ether-water were heavily contaminated with other constituents of the leaf cells.

The recent improvements in extracting leaf protein are due chiefly to the work done by Chibnall and his co-workers, Chibanll (1923, 1924) tried to remove the vacuolar protein, which is soluble in water, by cytolysing with ether and pressing between filter cloth; and after grinding, to get rid of the cell wall material by squeezing through silk gauze and of the chloroplasts and nuclear material, which are dispersed in colloidal solution, by filtering through paper pulp. The cytoplasmic protein, which is obtained in true solution and left in the clear filtrate, was precipitated by the addition of acid. By this method, Chibnall obtained from spinach leaves a preparation of cytoplasmic material consisting 96.6% protein. After failing to get good results in some other cases in preparing protein by the use of ether, Chibnall et

al. (1933) used "used" ether-water for extracting protein from cocksfoot and got a sample of protein containing 13.9% of nitrogen which represents 19.1% of the total grass-nitrogen.

Foreman (1938) improved on the ether method in preparing protein by washing the cytolysed leaf redidues with saturated aqueous ammonium sulphate before grinding. The nitrogen content of the dried and ash-free preparations obtained by Foreman from perennial rye-grass and white clover varied between 11.32% and 14.73%.

The yield of the proteins varied from plant to plant. The cause is rather complex. In some cases, the variation might be attributable to the very high (H^+) of the vacuole fluid in theliving cell, as explained by Chibnall (1939), so that after cytolysis, it brings all the cell contents to between pH 5 and pH 4, the isolelctric point of proteins, and thus renders the leaf protein insoluble. Foreman (1938) suggested that the protoplasm of leaf cell contains two types of "complexes": in "complex a" the protein is in loose combination with phosphates of potassium or sodium and can be dispersed into solution when the (H^+) in the vacuolar fluid is low and vacuole

phosphate is absent. In "complex b", the protein is in loose combination with phosphates of calcium or magnesium and remains insoluble in the presence of vacuolar sap or of pure water. According to this suggestion the yield of ~~soluble~~ soluble protein is determined by the content of these two types of complexes, and it is possible that part or even the whole of the cytoplasmic protein may be rendered insoluble through base exchange after cytolysis.

More recently Lugg (1939) prepared proteins from plant leaves in various ways by grinding leaves with different solvents, namely sodium phosphate buffer of pH 7.2, alcohol-ether buffer mixtures pH 9.2, water, and borate buffer pH 9.2. He obtained some "extracted" protein preparations from cocksfoot and rye-grass containing 12.5 to 14.6% of nitrogen, and favoured the use of a mildly alkaline buffer in order to disperse the protein-containing cell units into the juice.

PRELIMINARY INVESTIGATIONS. (a) EFFECT OF ANAESTHETIZING, FREEZING AND GRINDING ON THE CELL WALL OF GRASS LEAVES.-In order to extract the protein from fresh leaves, the cell wall of the leaf should be broken or rendered permeable upon which the success and completeness of extraction depend. To this effect, some

rye-grass leaves were treated in the following different ways and their effects on the cell wall were examined under the microscope.

(1) A slice of fresh leaf without any treatment was made for comparison.

(2) Frozen in solid CO_2. Holes were bored with a hot iron bar in a block of solid CO_2. Some small bunches of grass were put in the holes and covered with CO_2 powder. After having been left in the solid CO_2 for ten minutes at about -79°C the grass was taken out. Under the microscope, the cell wall appeared to be practically intact and did not show any significant difference from that without any treatment under (1)

(3) Ground grass. Bunches of grass were ground in a mincing machine, and in a mortar together with sand ~~separately~~. Most of the cells, in both cases, were broken and some of the chloroplastides were liberated. The degree of disintegration, of course, depended upon the thoroughness of grinding.

(4) Frozen in CO_2 and then ground. Most of the cells were broken showing similar condition to those in (3).

(5) Frozen in liquid oxygen. The liquid oxygen was kept in a thermos flask, and a small bunch of grass

was immersed in it for ten minutes. The temperature in liquid oxygen is about -182.5°C. Some of the cells were broken. It showed that freezing in liquid oxygen did cause some disintegration of the cell wall but was still not so effective as grinding.

(6) Treated with "fresh" ether-water. Some grass leaves were immersed in "fresh" ether-water for ten minutes and then examined under the microscope. The chloroplastides were collected near the cell wall showing that there had been plasmolysis.

(7) Treated with "used" ether-water. The ether-water left from (6) was used for this purpose. Another bunch of grass was immersed in it for ten minutes. It showed signs of plasmolysis as in (6) but to a less extent.

From the above observations, it is obvious that grinding either by hand or by machine is the most effective method of breaking the cell wall of grass leaves.

(b) ISOELECTRIC POINT.-The isoelectric point of grass proteins is generally accepted as being pH 4.5 (Chibnall et al. 1933; Lugg, 1938). To confirm this, the following investigation was made. Samples of fresh herbages were ground twice in a

mill with three times their weight of borate buffer solution of pH 9.2. The mashes were squeezed in a linen bag and the colloidal extracts were filtered at the pump. Seven 10 ml. portions were taken from each extraction and poured into test tubes. To each portions, a definite amount of N/10 H_2SO_4 was added. After standing, the volumes of protein preparation in the different tubes were compared and their pH values were determined and are recorded in the following table:

Table XII.

Tube No.	N/10 H_2SO_4 added (ml.)	pH value			
		W.C.	C.	R.	T.
1	7.0	5.04	5.20	5.16	5.12
2	7.5	4.78	5.00	4.85	4.90
3	8.0	4.46	4.56	4.52	4.50
4	8.5	4.22	4.30	4.17	4.20
5	8.8	4.00	4.10	4.02	3.98
6	9.0	3.80	3.92	3.88	3.81
7	9.2	3.56	3.74	3.61	3.58

By simple inspection, the extracts, to which 7.5 to 8.5 ml. of N/10 H_2SO_4 had been added, gave the largest precipitates having pH values from 5.00 to 4.20 which might be regarded as covering the isoelectric range of the leaf proteins.

PROCEDURE IN PREPARING LEAF PROTEIN. The method used in the present investigation for preparing leaf protein was more or less based on Chibnall's method (1923, 1924) but a sodium borate buffer solution was used as suggested by Lugg (1939).

Three to four hundred g. of fresh grass were macerated twice in an electric mincing machine with three times its weight of a borate buffer solution of pH 9.2, containing 1.1 g. of boron per litre. The debris of cell wall was removed by squeezing the pulp in a linen bag, and the colloidal extract thus obtained was filtered with suction on a Büchner funnel. To the filtrate an adaquate amount of 2N H_2SO_4 was added (about 4 ml. of 2N acid for 100 ml. of extract) to precipitate the protein at its isoelectric point. After standing over night, the supernatant liquid was syphoned off. The flocculated protein was washed twice with water, and after settling the supernatant liquid was again syphoned. The protein suspension was then centrifuged at 3000 r.p.m. for three minutes and purified by extracting four times with absolute alcohol and twice with methylated ether in the centrifuge tubes. Since a protein preparation thus obtained still contains some lipoids which cause

Table XIII. (see Table V facing page 29 for particulars of the samples.)

% of N in fat free protein preparations				N in fat free protein preparations as % of total leaf-N			
W.C.	C.	R.	T.	W.C.	C.	R.	T.
10.46				38.11			
10.15				X16.17			
10.24				30.14			
11.08				33.56			
13.83		10.40		X 6.07		24.47	
11.65	11.01	6.00		30.60	25.83	25.43	
		8.09				29.60	
11.89	11.16	10.75		27.42	20.65	26.49	
10.03		11.96		30.63		21.69	
		10.20				32.22	
		11.01				33.68	
10.88	9.69	9.60		27.78	19.63	25.08	
13.19	10.11	10.27		24.03	22.73	28.31	
	8.57	10.72			19.88	26.80	
12.84				37.03			
	10.57				24.07		
	9.94				17.14		
	9.45				34.04		
	11.03				26.06		
	11.62				31.99		
	11.63				20.62		
	10.89				26.68		
	10.41				15.10		
	12.72				28.83		
	12.85				22.16		
	14.11				25.93		
		9.98				20.62	
		8.57				21.90	
		12.45				28.56	
13.78		13.12		42.55		24.28	
12.99		12.25		31.86		25.29	
		12.75				23.78	
			10.70				30.62
			11.13				X19.68
			8.55				30.59
			11.76				34.20
			10.81				24.72
			12.54				28.64
			8.00				X 9.94
			8.54				33.36

X Break of centrifuge tubes caused some loss of protein.

Table XIV. Grasses under different manurial treatments.
(see Table VI facing page 29 for particulars of the samples)

% N in fat free protein preparations	N in fat free protein preparations as % of total leaf-N
Cocksfoot	
11.13	27.93
11.01	33.87
Rye-grass	
11.04	29.48
11.25	26.18
Cocksfoot	
10.49	20.54
10.29	31.07
Rye-grass	
11.39	20.39
11.75	23.66
Rye-grass	
10.85	32.20
11.96	37.27

trouble in determining the sulphur-containing amino acids, it was necessary to subject it to further extraction with petroleum ether (40°-60°) in a Soxhlet apparatus for twenty hours. It was then dried in a vacuum desiccator over P_2O_5. The protein preparation extracted in this way, though consisting mainly of cytoplasmic protein, is really a mixture of many proteins having the same solubility. The extractions were made at room temperature.

NITROGEN-CONTENT OF PROTEIN PREPARATIONS AND THE EXTENT OF EXTRACTION. The nitrogen-content of the protein preparations was determined by the Kjeldhal method as described on page 28, and is recorded in Table XIII and Table XIV on the opposite pages.

From the nitrogen-content of the protein preparations, the extent of extraction, which was expressed as "N in fat free protein preparations as % of total leaf-N", was calculated according to the following formula:

$$\frac{\text{Wt. of protein} \times \%\ \text{N in protein} \times 100}{\text{Wt. of fresh sample} \times \%\ \text{D.M.} \times \%\ \text{N in dry sample}}$$

= % of total leaf-nitrogen as protein-nitrogen.

Since it was unavoidable that there should be some loss of material during the different stages of extraction, the figures calculated give only a

general idea of the extent of extraction; and in a few cases, the figures are exceptionally low due to the breakage of centrifuge tubes.

SECOND EXTRACTION. In order to see whether there were any differences in the chemical composition between these extracted protein preparations and the protein left in the residue, some second extractions were made from the grass residues left after the first extraction. The grass residues left after being squeezed was thoroughly ground in a mortar with some sand and an appropriate amount of the sodium borate buffer solution. The debris was then squeezed through a linen bag and the extract was treated in the same way as the first extraction. The pH value of the second extract was about 9.0 and higher than that of the first extract, which was about 8.5; therefore the second extract needed more acid to bring the pH to the isoelectric point. The second protein preparations were very small, weighing about 1/4 to 1/6 of the first preparations, and only the total-nitrogen and amino-nitrogen were determined and are recorded in the following table together with the total-nitrogen and amino-nitrogen contents of the corresponding first protein preparations.

Table XV.

Sample No.	Species	Extraction	Total-N in fat free protein preparation (%)	% of total-N in protein as amino-nitrogen
55	Rye-grass	1st.	11.75	63.3
"	"	2nd.	10.40	63.0
57	"	1st.	11.95	56.7
"	"	2nd.	9.83	56.7

Since only a few samples were obtained by second extraction, it is impossible to draw a definite conclusion with respect to their nature, but as shown by the above table there seems to be no striking difference between the extracted protein and the protein left in the residue so far as the amino-nitrogen is concerned. The nitrogen-content in the protein preparation depends on the process of extraction rather than upon the nature of the protein.

DISCUSSION. The nitorgen-contents of the protein preparations appeared to be rather irregular. The figures for White clover varied from 10.0% for a sample at flowering taken in June 1939 to 13.8% for a young sample which had been grazed and was taken in June 1940. Those for Cocksfoot varied from 8.6% for an old aftermath sample taken in October 1939 to 14.1% for a sample at heading taken in June 1940.

In the case of Rye-grass, the results varied from 8.1% for an aftermath sample which contained some old stems and was sampled in August 1939, to 13.1% for a sample at heading which had been grazed by sheep and was taken in June 1940. The nitrogen-content of the protein preparation from an old Rye-grass sample taken in August 1939 was exceptionally low, being only 6.1%. As to the protein preparations of Timothy, the highest nitrogen-content was 12.5% for an aftermath sample which consisted mostly of stalks and was taken in September 1940, and the lowest figure was 8.0% for a young sample which contained some decayed leaves and was sampled in June 1939.

The extent of extraction, in general, seems to depend upon the following factors: (1) The grinding operation, upon which the thoroughness of disintegration depends. (2) The pH value of cell sap. In some cases, for example, the (H^+) of the vacuole fluid in rhubard is so high (about pH 4.0) that, after cytolysis, it may bring the cell contents to the isoelectric point of protein. But in most cases, the pH values of the leaf cell sap of various plants are alkaline with respect to their isoelectric point (Chibnall and Gorver, 1926). (3) The nature of the protoplasm of

leaf cell, that is whether the protein in the leaf cell is in combination with phosphates of potassium or sodium and so is easily dispersed into solution; or in combination with phosphates of calcium or magnesium and is insoluble. (4) The condition of the sample. Old and fibrous samples, being more difficult to disintegrate, will naturally produce less protein. In the present investigation, since all the samples were treated in the same way and the use of a sodium borate buffer solution tended to reduce the effect of the pH value of the cell sap and that of the nature of protoplasm, the most dominant factor affecting the extent of extraction was the condition of the samples.

White clover, being less fibrous, produced the most protein of the four species. The extent of extraction for White clover varied from 24-43%. Cocksfoot produced the least protein, being from 15-34%. Rye-grass and Timothy gave similar results. The figures were from 21% to 34% for Rye-grass and from 25% to 34% for Timothy. Within each species, young and aftermath samples generally produced more protein than the old ones as shown in Table XVI, in which the figures for all four species are summarized according to the condition of the samples.

Table XVI.

Condition of sample	N in protein preparation as % of total-N			
	W.C.	C.	R.	T.
Young	37-38	29	21	---
Flowering or heading	28-31	22-26	22-25	25-31
Old	24-31	17-21	22-25	---
Aftermath	27	15-26	27-34	29-33
Grazed	32-43	32-34	26-29	31-34

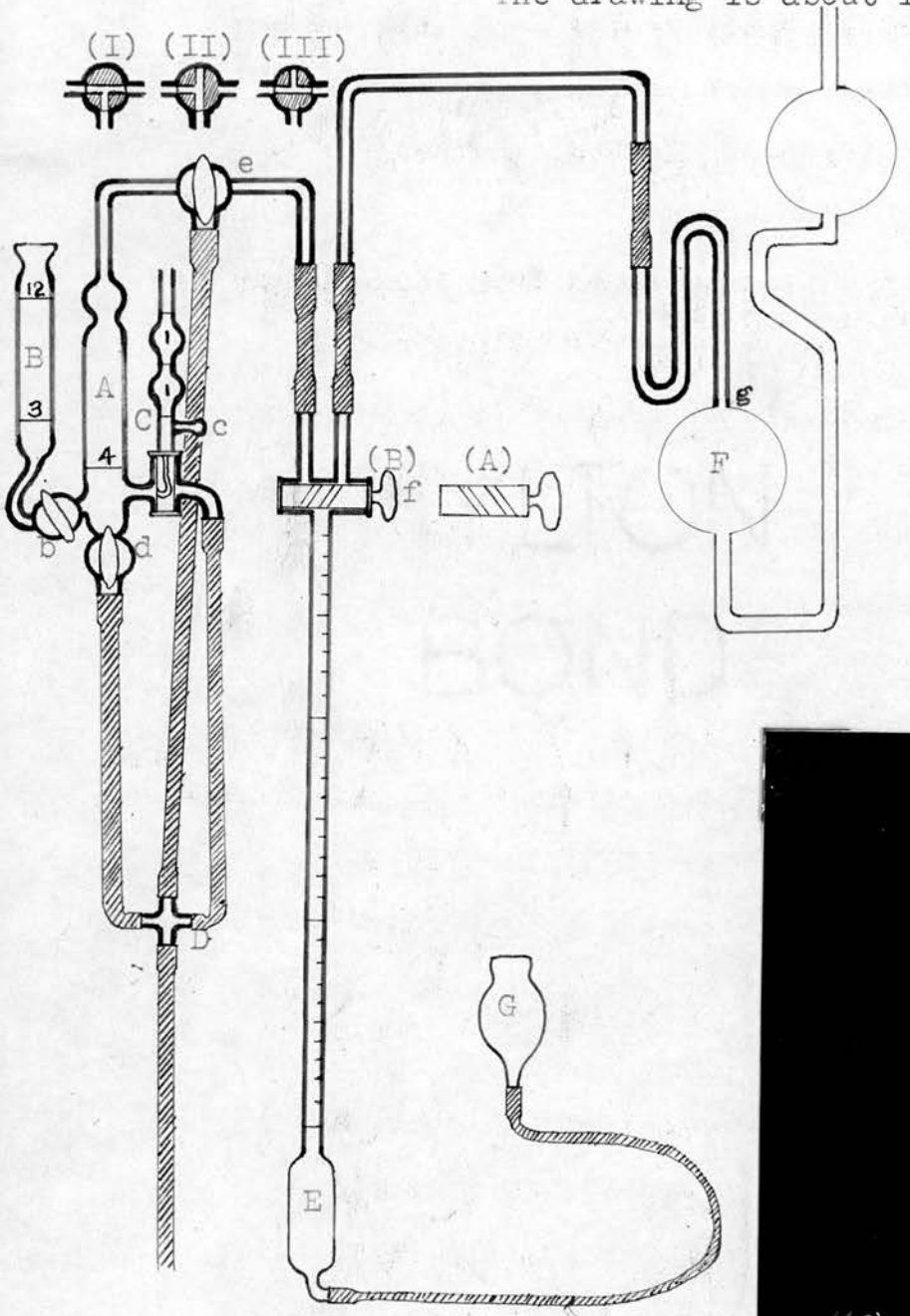
There are, however, a few cases there the results were rather irregular. The figure for a White clover aftermath taken in September 1939, sample 35, was only 27%, even lower than those of some old samples; again sample 3, young Rye-grass which had only a few leaves left and was taken in June 1939, gave a figure of 21%, being the lowest among all the figures for Rye-grass.

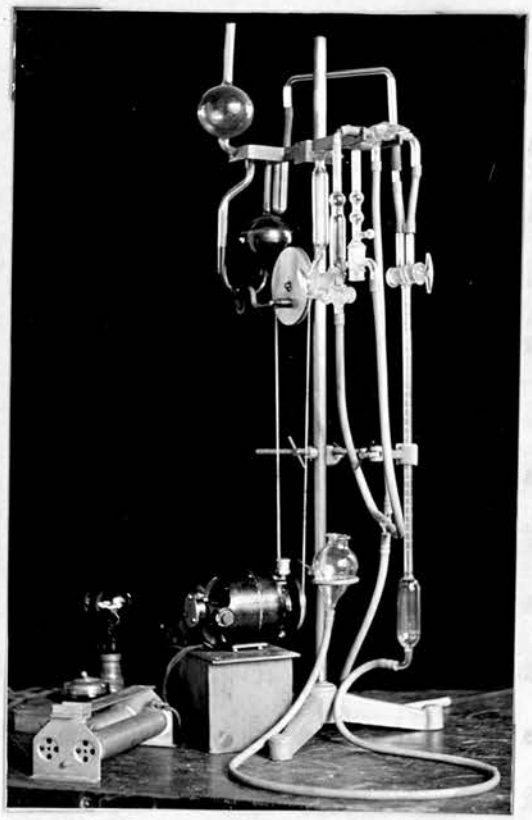
AMINO-NITROGEN OF THE PROTEIN PREPARATIONS

METHODS OF DETERMINING AMINO-NITROGEN AND DETAILS OF TECHNIQUE. The amino-nitrogen of proteins is generally determined either by the copper method or by ~~the~~ Van Slyke's gasometric method. The copper method, suggested by Kober and Sugiura (1912, ~~1913~~) and Kober (1917) for determining α-amino-nitrogen, was based upon the formation of copper salts through the reaction between the amino-acid or digest material and excess copper present. It was modified by Utkin (1933) for the determination of amino-nitrogen in protein decomposition products, and was further improved by Pope and Steven (1939) to determine the amino-nitrogen in enzymic digests of proteins. The accuracy of the method is easily determined for pure amino-acids, but cannot be determined directly for more complex compounds. The Van Slyke method (1911; 1911-1912; 1912, 1; and 1915), which was adopted in the present investigation, is based upon the principle that the aliphatic amino-groups react with nitrous acid with the liberation of nitrogen gas. Since the improvement of the Van Slyke apparatus, the accuracy of measurement has been so increased that only one or two ml. of the hydrolysed solution are required for analysis.

Fig. VIII. Showing the Van Slyke micro-apparatus.

The drawing is about 1/4 of the actual size.





(a) PRINCIPLE OF THE METHOD.-Free aliphatic amino-groups react with nitrous acid with the liberation of nitrogen gas according to the following equation:

$$RNH_2 + HNO_2 = ROH + N_2 + H_2O$$

Half of the nitrogen gas produced comes from the nitrite so that the nitrogen gas evolved should be divided by two in order to get the weight of amino-nitrogen.

When sodium nitrite reacts with glacial acetic acid, nitrous acid is liberated and, in solution, decomposes into nitric oxide and nitric acid:

$$3HNO_2 \rightleftharpoons HNO_3 + 2NO + H_2O$$

The nitric oxide is used to displace the air in the apparatus. After the introduction of the amino-acid solution, the nitrogen gas evolved is mixed with the nitric oxide. The latter is absorbed by alkaline potassium permanganate solution and the pure nitrogen left is measured in a gas burette.

(b) APPARATUS.-In the present investigation, a Van Slyke's micro-apparatus was used. The structure of the apparatus is shown in the accompanying Fig., heavy lines being used to show capillary tubing. The deamination process is carried out in the vessel A having a capacity of about 20 ml. It is connected with the tubes B and C by the stop-cocks b and c at

opposite sides and with the out-flow tube D by the cock d at the bottom. The tube B, which is marked at the volumes of 3 ml. and 12 ml., is used for the addition of glacial acetic acid and sodium nitrite. The tube C, which has a volume of 2 ml. and is graduated, is used for adding the amino-solution, The tube D is for running off the reaction mixture after each determination. The deamination vessel A has a bulb at the top and a constriction as shown in order to prevent spirting of the reaction mixture during shaking. By means of the capillary tubes, the deamination vessel is connected with the gas burette E. They are separated with a three-way cock e and cock f. The gas burette consists of a long tube with a bulb at the lower end. The tube is 3 ml. capccity and is graduated into 1/100's of a ml. The bulb provides a volume of about 20 ml. for holding the mixture of N and NO gases. The end of the bulb is connected to the funnel G by means of a long rubber tube. Both the bulb and the funnel are filled with distilled water and by moving the funnel the water and gas can be driven to & from the gas burette. The gas burette is, on the other side, connected to a Hempel pipette which is about 2/3 filled with alkaline potassium permanganate for absorbing NO. Between the

deamination vessel and the Hempel pipette, a driving wheel is fixed at a suitable position so that the driving rod can be connected with the deamination vessel or the Hempel pipette. The driving wheel is turned by a small electric motor which is fixed on the bench below the wheel and connected with a resistance to regulate the speed.

(c) REAGENTS.-(1) Glacial acetic acid.

(2) Alkaline potassium permanganate solution prepared by dissolving 50 g. of $KMnO_4$ and 25 g. of KOH in one litre of water.

(3) Thirty percent $NaNO_2$ solution. Since commercially pure $NaNO_2$ yields more or less nitrogen gas, a blank test should be made for each new stock of $NaNO_2$.

(d) HYDROLYSIS.-About one g. of the grass protein preparation is dissolved in 25 ml. of 3N HCl, and boiled at about 110°C in a conical flask under a reflux for eight hours. A preliminary investigation was carried out on the length of time required for hydrolysis, which will be described latter. After hydrolysis, the amino-solution is poured into a 100 ml. flask, neutralized with KOH (because amides, urea and the guanidine group of arginine, under the usual conditions in acetic acid,

do not react to a measurable extent but react in mineral acid solution), and made up to the volume by adding distilled water. Since 2 ml. of the hydrolysed solution are used for each amino-nitrogen determination, the concentration of the solution should be diluted to such an extent that the nitrogen gas evolved, including the blank-nitrogen, is about 2-3 ml.

(e) MANIPULATION.-(1) Displacement of air. Turn the cock f to the position (A) and the cock e to the position (I). Fill the whole burette and the capillary tubes as far as to the cock e with distilled water by raising G. Disconnect E and A by turning e to the position (II). Into A, through B, pour first 3 ml. of glacial acetic acid and then 12 ml. of $NaNO_2$ by opening b and closing d and c. Then close the cock f and turn the cock e to the position (III). Shake A (the cock b is left open) until only 4 ml. of the solution are left in A. Turn the cock e to the position (II) again and the gas in A is released. To ensure a complete displacement of the air in A, the process should be repeated once more. After the second displacement, repeat the shaking till the solution in A is reduced to 4 ml. Close the cock b, and A and E are then connected by turning the cock e to the position (III)

and the cock f to the position (A).

(2) Decomposition of the amino-solution. Two ml. of the hydrolyaed amino-solution are introduced into A through C. At this time, b, c, and d are all closed; e is in the position (III); andf in the position (A). The deamination vessel is shaked at a constant speed of about 300-350 r.p.m. The period of shaking depends upon the nature of the amino acids. A preliminary test was made with glycine to find the adaquate length of time for shaking (see page 83). Since the amino acids and the hydrolysed grass protein do not foam very much, it is not necessary to add caprylic alcohol before shaking as suggested by Van Slyke (1912).

(3) Absorption of NO. After the deamination process has been finished, all the gas in A and in the capillary tubes connecting A and E are driven into E by pouring distilled water into B and lowering G. As soon as the solution reaches the cock f, shut f. If some liquid enters the burette tube, let it run down to E and join the distilled water originally present. Drive the mixed gas into the Hempel pipette by turning the cock f to the position (B) and raising G until the distilled water reached the end of the capillary tube at g leading to F, and the cock f is shut again, Lift

the driving rod from the shoulder of cock d and place the other hook over the horizontal lower tube of the pipette. Shake the Hempel pipeete for a few minutes to hasten the absorption until all NO is absorped and the volume of the nitrogen gas is constant.

(4) Measurement of nitrogen gas. The pure nitrogen gas left is driven back to E by turning the cock f to the position (B). The water bubbles in the capillary tubes should be got rid of and the nitrogen gas is carefully measured in the burette E after adjusting the water level in E and G.

(5) Other remarks. All the stoppers should be kept airtight. After each deamination, the deaminated solution is run out from D; and the deamination vessel A, tubes B and C, and the capillary tubes connecting A and E should be rinsed with water. The apparatus is then ready for use again.

(f) EXAMPLE OF CALCULATION.-The volume of the nitrogen gas in ml. is converted into weight in mg., from which the weight of blank nitrogen must be deducted, in the usual way and divided by two in order to get the weight of amino-nitrogen. A correction is made for the pressure and temperature. The amount of amino-nitrogen is expressed as "% of the total nitrogen in

the protein preparation", and is calculated according to the following formula:

$$\frac{\text{Wt. of amino-N} \times 100}{\text{Wt. of total N in 2 ml. sol.}} = \% \text{ of protein-N as amino-N.}$$

The method of calculation can be illustrated by the following example:

Weight of protein preparation	1 g.
% of N in protein preparation, say	12%
Volume of the hydrolysed solution	100 ml.
2 ml. solution contain	2.4 mg. of N
Volume of N, say (at 20°C, 760 mm. pressure)	2.5 ml.
Weight of N	1.4225 mg.
Blank, say	0.2 mg.
Weight of amino-N	1.2225 mg.

$$\frac{1.2225 \times 100}{2.5} = 48.9 \% \text{ protein-N as amino-N.}$$

PRELIMINARY INVESTIGATIONS. (a) TIME OF SHAKING.- Glycine was used for this purpose. About 0.3 g. of pure glycine (B.D.H.) was dissolved in 100 ml. of distilled water. Portions of 2 ml. of the solution were used each time for the amino-nitrogen determination, shaking for different lengths of time and at a speed of about 300-350 revolutions per minute. The results are shown in the following table:

Table XVII.

No. of test	Percentage of recovery		
	5 minutes shaking	8 minutes shaking	10 minutes shaking
1	109.2	113.0	112.8
2	108.8	110.8	111.0
3	107.7	111.5	111.6
4	110.4	111.8	111.9
Average	109.03	111.77	111.83

From the above table, it seems to be that eight minutes shaking is sufficient to produce the maximum yield of amino-nitrogen gas. In some cases, ten minutes shaking produced more amino-nitrogen, but the average yield of ten minutes shaking is only 0.06% higher than that of eight minutes shaking, and such a different is quite negligible. The average recovery of amino-N from glycine was 111.8%. It is always higher than that theoretically calculated and rather ~~irregular~~ variable. Van slyke (1911) got a recovery of 103% for glycine, and Levene and Van Slyke (1912) got 112.4% recovery. This is probably due to the fact that glycine gives off not only nitorgen but also CO_2 and some other gas.

(b) AMINO-NITROGEN DETERMINATION WITH PURE AMINO ACIDS.--Some pure amino acids, namely arginine, cystine, histidine, lysine and tyrosine, were used to determine their amino-nitrogen contents. Since cystine and

tyrosine are sparingly soluble in water, they were first dissolved in a few ml. of dilute HCl and then made up to suitable volume. All the other amino acids are readily soluble in water. Of these five amino acids, cystine, lysine and tyrosine have 100% of their nitrogen as amino-nitrogen; while three fourths of the nitrogen in arginine (including the NH_2 group of the guanidine nucleus), and two thirds of that in histidine are non-amino-nitrogen, and do not react with nitrous acid. Portions of 2 ml. from each solution were used for amino-nitrogen determination and shaken for eight minutes at 300-350 r.p.m. The yields of amino-nitrogen are shown in Table XVIII.

Table XVIII.

Amino acid	Average % recovery of amino-N	
	Van Slyke (1911)	Present investigation
Arginine	99.8	102.3
Cystine	107.4	108.5
Histidine	98.2	103.0
Lysine	95.4	96.9
Tyrosine	102.1	102.4

All the figures found in the present investigation were higher than those of Van Slyke. This might be due to the different conditions of analysis. Van Slyke shook the deamination solution for five minuted with

lots of ten ml. each of the amino-solutions, while in this investigation shaking lasted for eight minutes and only two ml. each of the amino-solutions were used for analysis. The results for ~~cystine~~ arginine, histidine and tyrosine, as shwon above, were quite close to theoretical. But that for cystine was 8.5% higher, and that for lysine was 3.1% lower, than those calculated. Van Slyke (1911) explained that a slight amount of CO was apparently evolved from cystine together with nitrogen, causing an over-estimation. While the under-estimation for lysine was due to the fact that the nitrogen in lysine exists in two forms, one in α-position and the other in ε-position and the latter needs more time to react with nitrous acid than the former. According to this explanation, the under-estimation for lysine is probably due to insufficient shaking. Van Slyke also tried shaking the lysine solution for different lengths of time and found that the amino-nitrogen came out about 95.4% for five minutes and 100.3% for fifteen minutes at 24°C; and about 85.1% for five minutes, 92.0% for fifteen minutes and 98.0% for thirty minutes at 19°C.

(c) TIME OF HYDROLYSIS.-Portions of a grass protein preparation, and of grass protein and glycine

were each hydrolysed with 25 ml, of 3N HCl. After being hydrolysed for 4, 6, 8, and 10 hours, 2.5 ml. of the solution were pipetted out, neutralized with KOH, and made up to 10 m. with distilled water. After the removal of each portion, the hydrolysing flask, together with the solution left in it, was weighed; and the weight was again taken after the succeeding hydrolysis and before the next sample was drawn. The difference in weight due to the loss of water during boiling was made up by adding distilled water in order to keep the concentration constant. The hydrolysis was continued until the maximum yield of amino-nitrogen was reached. This showed what time was required for a complete hydrolysis. Portions of 2 ml. were used for amino-nitrogen determination and shaken for eight minutes at a speed of 300-350 r.p.m.

(1) Hydrolysis with grass protein preparation. About one g. of White clover protein, sample 18, was used for this purpose. The yields of amino-nitrogen after different periods of hydrolysis were as follows: Table XIX.

No. of test	% of protein-N as amino-N			
	4 hours	6 hours	8 hours	10 hours
1	62.8	71.9	73.2	73.2
2	62.3	71.8	72.9	73.0
Average	62.6	71.9	73.1	73.1

(2) Hydrolysis with grass protein preparation and glycine. About 0.5 g. of the White clover protein and 0.1 g. of pure glycine, which was used in the preliminary investigation for the time of shaking, were mixed and hydrolysed with HCl for different periods as (1). The yields, from which the amount of amino-N in the glycine added (111.8% as shown on page 83) has been deducted, are recorded in the following table: Table XX.

No. of test	% of protein-N as amino-N			
	4 hours	6 hours	8 hours	10 hours
1	63.4	71.0	73.0	73.2
2	63.8	72.6	73.4	73.3
Average	63.6	71.8	73.2	73.3

These two investigations agree quite well and show that the maximum yield of amino-nitrogen in grass protein can be obtained after hydrolysis for eight hours.

When proteins are hydrolysed with acid, both amino-nitrogen and ammonia increase and the end-point of hydrolysis is reached when the amino-nitrogen reaches a maximum with the least possible formation of ammonia. The time required for complete hydrolysis depends upon the temperature, at which the hydrolysis is carried out, and the concentration of acid.

Table XXI.

% of total-N in protein preparation as amino-N			
W.C.	C.	R.	T.
66.4			
71.2			
73.1			
65.5			
64.6		61.1	
67.8	63.6	53.2	
		57.2	
67.5	64.2	58.2	
58.6		66.1	
		59.2	
		59.3	
62.9	71.8	68.0	
71.7	60.7	57.3	
	70.2	61.6	
68.4			
	60.0		
	63.2		
	63.0		
	57.3		
	62.8		
	63.2		
	62.4		
	64.1		
	61.2		
	62.3		
	62.6		
		60.3	
		63.4	
		62.8	
60.6		58.0	
69.6		62.2	
		60.2	
			64.1
			66.5
			62.4
			62.3
			62.4
			56.9
			57.6
			56.8

Henriques and Gjaldback (1910) concluded that complete hydrolysis was reached by heating with 3N HCl in an autoclave at 150°C for one and half hours. Van Slyke (1912, 2) found that hydrolysis could be completed by heating with 20% HCl at 100°C for 24 hours. In this investigation, 3N HCl, which boils at about 110°C, was used and eight hours boiling appeared to be sufficient to effect a complete hydrolysis.

RESULTS AND DISCUSSION. The amino-nitrogen contents, expressed as percentage of the total nitrogen in the protein preparation, are recorded in Tables XXI and XXII. Particulars of the samples are given in the tables facing page 29.

As shown by Table XXI, there are no striking differences between the four species with regard to their amino-nitrogen contents. White clover contained comparatively more amino-nitrogen, Cocksfoot came next, and Rye-grass and Timothy had the least amino-nitrogen. The figures for White clover varied from 58.6% to 73.0%, those for Cocksfoot from 57.3% to 71.8%, those for Rye-grass from 53.2% to 68.0%, and those for Timothy from 56.8% to 66.5%.

In considering the amino-nitrogen in each species, it will be seen from Table XXIII that it tended to

increase as the grass was getting ripe. This is particularly remarkable when comparison is made between these samples taken from the same farm at different stages of growth, and all the four species showed more or less the same variation with a few exceptions. For example, the figure for a Rye-grass sample, No. 21, which was old with a few seeds left and taken in August 1939, was lower than that for sample 6, which was heading and sampled in June 1939 from the same field. Again, a Timothy sample, No. 59, which was heading and taken in July 1940, had a figure about the same as that for a young sample, No. 46, which was sampled in June 1940 and has been grazed.

Table XXIII.

Condition of sample	% of total-N in protein preparation as amino-N			
	W.C.	C.	R.	T.
Young	65-68	61	60	57
Flowering or heading	59-71	57-63	60-68	62-64
Old	68-73	68-72	53-63	67
Aftermath	66-68	61-64	57-62	57-57
Grazed	61-70	63-63	58-63	62-62

Aftermath generally appeared to contain a higher amino-nitrogen content than those samples at about the same stage of growth but taken earlier in the season

Table XXII. Summary of amino-N contents of grasses under different manurial treatments.

	% of total-N in protein preparation as amino-N
Cocksfoot	
	58.3
	59.0
Rye-grass	
	63.1
	54.8
Cocksfoot	
	60.3
	62.3
Rye-grass	
	57.9
	63.3
Rye-grass	
	64.7
	56.7

from the same farm; and in some cases, the amino-nitrogen content in aftermath was not unlike that in ripe samples.

As to the effects of different manurial treatments on the amino-nitrogen content, Table XXII shows that the application of slag produced different effects on the amino-nitrogen contents in Rye-grass and Cocksfoot. The figure for Cocksfoot was lower in the sample taken from a plot receiving less slag than that for a sample from a plot receiving more slag, and the reverse was the case for Rye-grass. Lime increased the amino-N contents in both Cocksfoot and Rye-grass. Ammonium sulphate inversely affected the amino-nitrogen content of Rye-grass.

In order to investigate the effect of season, the results are summarized in Table XXIV.

Table XXIV.

Month	% of total-N in protein preparation as amino-N			
	W.C.	C.	R.	T.
May	---	61-62	---	---
June	59-66	60-63	58-66	58-62
July	63-73	57-72	63-68	62-66
August	68-72	61-64	53-59	57
September	66-70	63-64	58-62	57-62
October	---	70	62-63	---

As shown by the above table, all the four ppecies

invariably contained less amino-nitrogen in May and June, reached the maximum during the month of July, and dropped a little as the season advanced.

SULPHUR-CONTAINING AMINO ACIDS AND INORGANIC SULPHUR OF PROTEIN PREPAR/TIONS

INTRODUCTION. Up to the present, cystine and methionine are the only amino acids known to contain sulphur. Cystine was discovered in the year 1899 by Mörner in the protein hydrolysates of horn. It is widely distributed in various proteins of both animal and vegetable sources, such as scleroproteins, lactalbumin, egg albumin, serum albumin, edestin, squash seed globulin, glycinin, wheat gliadin, glutelin, etc. Practically all the sulphur of wool, feather, horns, hoofs, claws, nails, and hair exists in the form of cystine.

Although as long ago as 1882-83, Danilewsky suggested that protein sulphur exists in two different states of combination, it was not until 1921-22 that the isolation of a second sulphur-containing amino acid, methionine, was carried out by Mueller from casein. Barger and Coyne (1928) proved the constitution of the new amino acid and suggested for it the name "methionine" after consulting with Mueller. Methionine exists in lactalbumin, egg albumin, caseinogen, vitellin, muscle proteins, edestin, glycinin, gliadins and some other proteins. Generally, most of the sulphur in

soluble proteins is in the form of methionine and that in the keratins and other insoluble proteins is mostly in the form of cystine.

With regard to the importance of these two sulphur-containing amino acids to animal life, many experiments have been carried out by different workers. Willcock and Hopkins (1907) found that the presence of cystine in the diet of mice was beneficial to their gorwth. Osborne and Mendel (1915) found that cystine is essentail to rats. Johns and Finks (1920) found that rats, fed on cooked phaseolin, grew more quickly when the diet was supplemented with cystine. Sherman and Merrill (1925)also found that cystine is an essential amino acid. Since the discovery of methionine, however, many investigations have shown that methionine is also essential and even that cystine can be replaced by methionine without any deteriorating effect on the growth of animals. Weichselbaum, Weichselbaum and Stewart (1932) showed that *dl*-methionine is as efficient as *l*-cystine. Jackson and Block (1931, 1932) found that methionine is indispensable in a diet which is deficient in cystine. Brand, Cahill and Harris (1935) suggested that cystine can be synthesised from methionine. This was confirmed by Womack, Kemmer and Rose (1937).

The indispensability of methionine and the synthesis of cystine by the living organism have received further confirmation from Rose (1938), Beach and White (1939), and Tarver and Schmidt (1939). On the other hand, Marston (1935) observed that dietary supplements of cystine, but not methionine, stimulate the growth of wool on a sheep maintained on a low-protein diet; Weichselbaum (1935) found a peculiar syndrome induced in rats by subsistance on a cystine-deficient diet, recovery from which could be secured by administration of cystine, but not of methionine; Madden et al. (1939) showed that in the regeneration of plasma protein in dogs, after artificial depletion, dietary methionine cannot take the place of cystine. From the foregoing review of the various investigations, it is impossible, for the time being, to draw a final conclusion in regard to the indispensability of these two amino acids. Since cystine and methionine are the principal sources of sulphur available to the organism, they must be the precusors of the various sulphur compounds of the body.

CHEMISTRY. Cystine, or di-β-thio-α-amino-propionic acid, $C_6H_{12}O_4N_2S_2$, $S.CH_2CH(NH_2).COOH$ / $S.CH_2CH(NH_2).COOH$,

is a di-amino-di-carboxylic acid, soluble in dilute alkalies and mineral acids, very slightly soluble in cold water, and insoluble in acetic acid, absolute alcohol and ether. After boiling in strong alkaline solution, cystine decomposes and a part of its nitrogen is evolved as ammonia. During prolonged hydrolysis with acid, it can be converted into cysteine, or β-thio-α-amino-propionic acid, $C_3H_7O_2NS$, $HS.CH_2.CH(NH_2).COOH$, which is more soluble.

Cystine in solution, after reduction to cysteine with sodium sulphite, gives a blue colour when treated with phosphotungstic acid reagent; after being treated with sodium cyanide which also reduces cystine to cysteine, it gives a red colour on the additionoof sodium-β-naphthoquinone-4-sulphonate in the presence of sodium sulphite and an alkali; and when saturated with ammonium sulphate and then treated with sodium cyanide and a few drops of concentrated sodium nitroprusside solution, it produces an intense purple colour on making the solution alkaline with ammonia. These colour reactions have been used as the basis of colorimetric methods for the determination of cystine.

Methionine, or γ-methylthiol-α-amino-η-butyric acid, $C_5H_{11}O_2NS$,

$CH_3S.CH_2.CH_2.CH(NH_2).COOH$,

is a mono-amino-mono-carboxylic acid. It is, in common with all amino acids, soluble in dilute alkali and acid, and insoluble in absolute alcohol and ether. It can also be dissolved in cold water.

A colour reaction between methionine and cupric chloride in hydrochloric acid has been reported by Kolb and Toennies (1939). When a solution of cupric chloride in concentrated hydrochloric acid is added to methionine, a colour, varying from deep brown to pale yellow according to the concentration of methionine, is produced. This reaction appears to be specific for methionine among the natural amino acids but has not yet been developped into a quantitative method for the determination of methionine.

METHODS OF DETERMINING SULPHUR-CONTAINING AMINO ACIDS AND INORGANIC SULPHUR. For the determination of sulphur-containing amino acids and inorganic sulphur, several methods have been devised. The most commonly used ones are as follows:

(a) FOLIN-MARENZI METHOD.-This method for determining cystine was developed from the early Folin and Looney (1922) process by Folin and Marenzi (1929), and modified by Rimington (1930) and Lugg (1932). It

is based on the production of a blue colour when cysteine reacts with the phospho-18-tungstic acid reagent, "uric acid reagent", cystine being reduced to cysteine with alkaline sulphite.

(b) SULLIVAN METHOD.-This method, introduced by Sullivan (1926) for determining cystine, is based on the fact that cystine is reduced by sodium cyanide to cysteine, which produces a red colour when treated with sodium-β-naphthoquinone-4-sulphonate in alkaline sulphite solution.

(c) IODIMETRIC METHOD.-Okuda (1925) titrated cysteine in the presence of iodide in acid solution with iodate, taking the appearance of free iodine as the end point. He modified this method (1929) by adding to the acid cysteine solution an excess of iodine and titrating back with thiosulphate, the end point being observed by the decoloration of a chloroform layer of the iodine. Baernstein (1930), working on the same prinple, reduced cystine in HCl with Devarda's alloy and reoxidised the cysteine with excess of iodine in KI, estimating the excess iodine by measuring the increase in pressure produced by the evolution of nitrogen from hydrazine sulphite in the Van Slyke apparatus.

(d) DIFFERENTIAL OXIDATION METHOD.-This method was devised by Lugg (1938) in an investigation of grass proteins and is based on the finding of Blumenthal and Clarke (1935) that under certain conditions cysteine-sulphur is converted into sulphate by nitric acid while methionine-sulphur is not.

(e) BAERNSTEIN METHOD.-Baernstein (1932) devised a method for the determination of methionine by hydrolysing with hydriodic acid. It reacts with the methyl thiol group of the methionine present with the evolution of methyl iodide, which is absorbed in alcoholic silver nitrate solution and the excess of the latter is determined with potassium thiocyanate. Baernstein (1934) modified this method by substituting a mixture of potassium acetate solution in glacial acetic acid and bromine for absorbing the methyl iodide, which is oxidized to iodate. From the latter, the iodine is liberated with potassium iodide and HCl, and titrated with thiosulphate. This was further improved by Baernstein (1936, 1, 2) into a combined method for the determination of cystine, methionine, and sulphate. The determination of cystine is based on the Okuda method (1929) by oxidizing the cysteine, reduced from cystine, with iodine and titrating back

with thiosulphate. The methionine is determined, in addition to the volatile iodide method, by the non-volatile residue, thiolactone of homocysteine, which, in alkaline solution, can be oxidized to homocystine by sodium tetrathionate and the corresponding amount of thiosulphate formed is ~~reduced to~~ titrated with biiodate. The sulphate is reduced to H_2S and SO_2 which are retained by $CdCl_2$ and $BaCl_2$ as CdS and $BaSO_3$ and are determined by oxidizing with iodine. This method has been modified by Kassell and Brand (1938) and Lugg (1938), and developed for micro-analysis by Kuhn et al. (1939).

In the determination of cystine and methionine, the formation of humin at the expense of cystine, especially when carbohydrates are present, during the acid hydrolysis employed in the colorimetric methods and the differential oxidation method, leads to low results. In the Baernstein method, the liberation of volatile iodide from source other than methionine such as lipoids, carbohydrates, and retained residues of organic solvents might cause an over estimation. This, however, can be reduced to the minimum provided the other sources of volatile iodide are completely removed, and besides, the results from the volatile iodide method

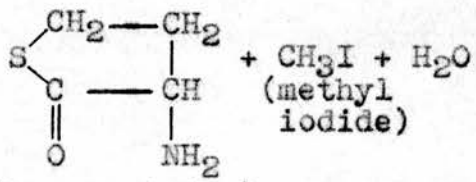
can be checked by the determination of homocysteine. Baernstein's method seems to be superior to other methods in rapid hydrolysis, the elimination of humin, and all the sulphur fractions can be determined by one hydrolysis.

METHOD ADOPTED AND DETAILS OF TECHNIQUE. In the present investigation, the procedure for determining the sulphur distribution was based on the Baernstein method.

(a) PRINCIPLE OF THE METHOD.-During the hydrolysis with HI, methionine is demethylated with the formation of the lactone of homocysteine and the liberation of methyl iodide; cystine is reduced to cysteine; and inorganic sulphate is reduced to H_2S and SO_2, while a small amount of H_2S is also formed from cystine and methionine. The reactions taking place during hydrolysis can be expressed by the following formulas:

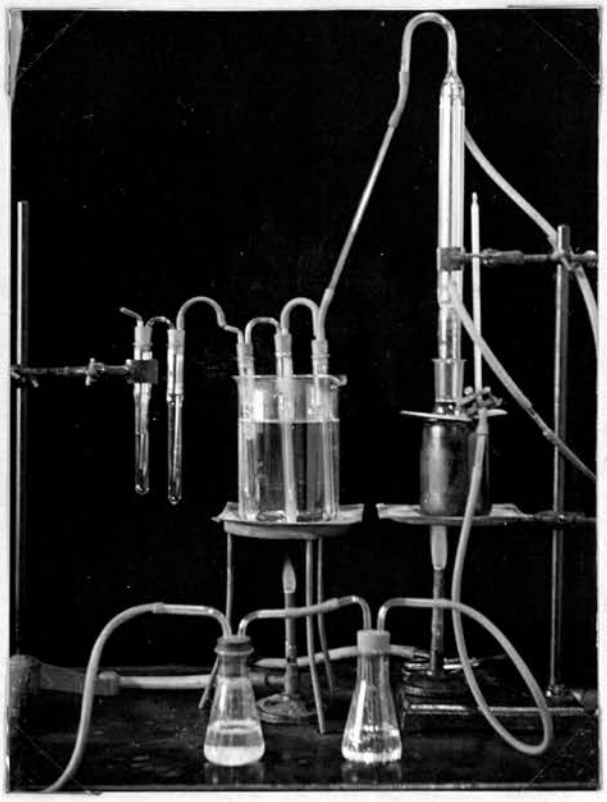
(1) $CH_3S.CH_2.CH_2.CH(NH_2).COOH + HI \longrightarrow$

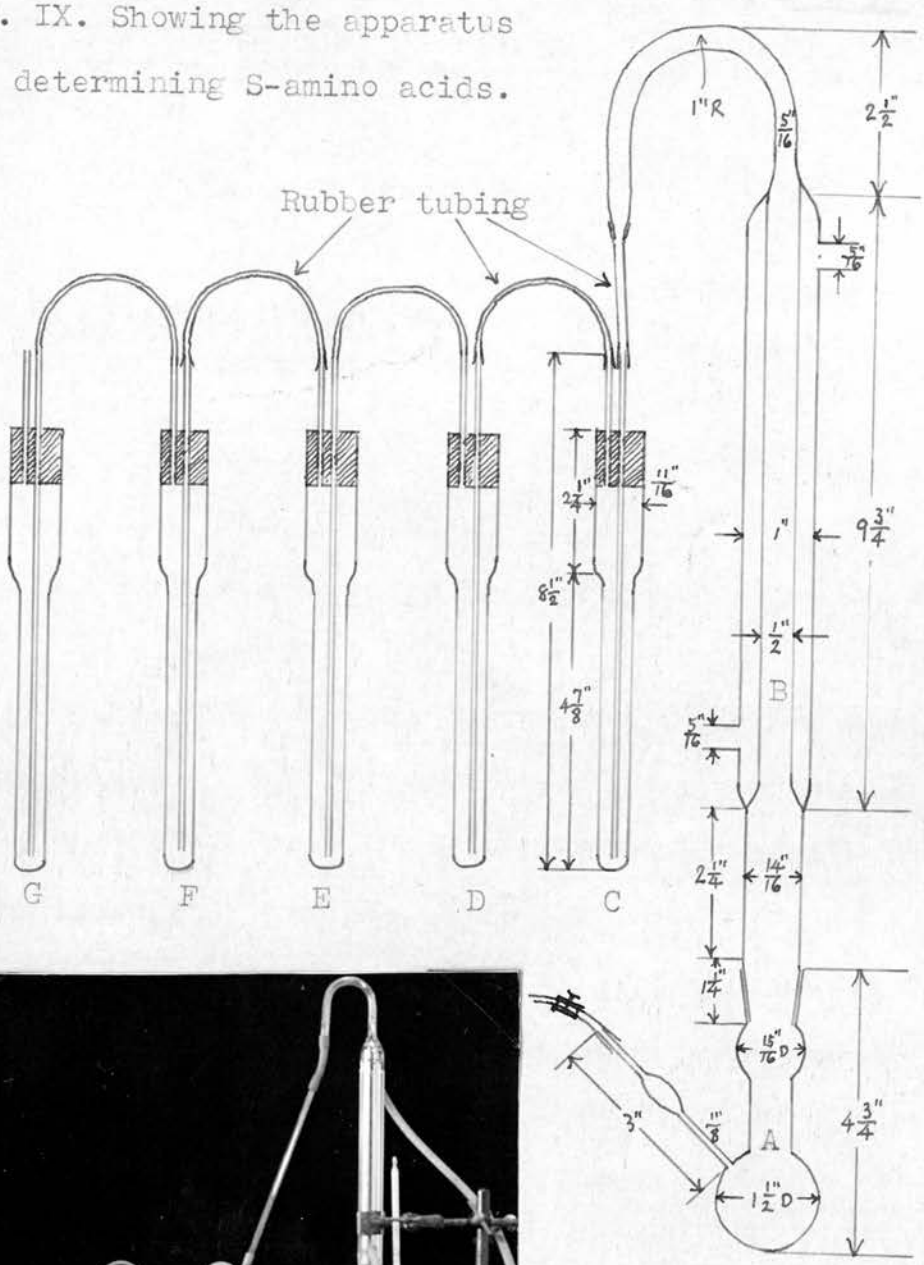
(methionine)



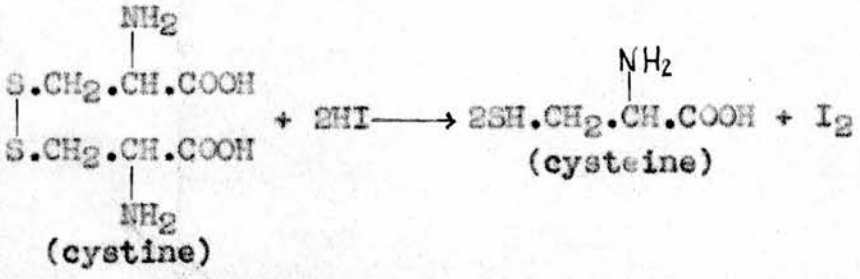
(homocysteine)

Fig. IX. Showing the apparatus for determining S-amino acids.





(2)



(cystine) (cysteine)

The volatile methyl iodide is absorbed in glacial acetic acid containing 10% of potassium acetate and a few drops of bromine. The iodine is liberated by adding KI and HCl and titrated with thiosulphate. As to the lactone ring of homocysteine left in the digest, it can be opened in alkaline solution and oxidized to homocystine by sodium tetrathionate and a corresponding amount of thiosulphate is formed. The thiosulphate is titrated with biiodate.

The cysteine reduced from cystine or originally present is in the digest. It is oxidized back to cystine by a known amount of biiodate, and the excess of the latter is titrated back with thiosulphate.

H_2S and SO_2 are absorbed by $CdCl_2$ and $BaCl_2$ as CdS and $BaSO_3$, which are oxidized with iodine by adding biiodate, KI and HCl.

(b) APPARATUS.-Baernstein's apparatus (1936) was used with a few modifications as shown on the attached Fig. It consists of a digestion flask A, condenser B,

and series of absorbing tubes. The digestion flask is connected to the condenser by a gound joint which should be wetted before joining. The joint is tight enough to prevent any leak. A mercury seal consisting of a rubber stopper with a glass sleeve as suggested by Kassell and Brand (1938) seems to be unnecessary. The absorbers are open at the top and connected in series by rubber stoppers. In addition to the four absorbers used by Baernstein, one more absorber containing red phosphorus suspended in water is introduced to remove any I and HI which might come over from the digestion flask. The absorber C contains 10 ml. of 1% red phosphorus suspended in distilled water. Its outlet tube is packed with glass wool to prevent the red phosphorus from passing into the next tube. The absorber D contains 10 ml. of a solution containing 20% $CdCl_2$ and 20% $BaCl_2$ for absorbing H_2S and SO_2. The absorber E contains 10 ml. of saturated $HgCl_2$ for removing the phosphine from hypophosphite. The absorber F contains 10 ml. of 10% potassium acetate solution in glacial acetic acid with six drops of bromine to absorb the methyl iodide. The absorber G contains 5 m. of the same solution as in the absorber F to ensure complete absorption of the volatile iodide.

(c) REAGENTS.-(1) HI, sp. gr. 1.7, is distilled twice in a stream of nitrogen and preserved by 1% potassium hypophosphite. The blank determination of HI is made at weekly intervals. The figures for blank determinations of 10 ml. of HI corresponded to about 0.02-0.04 mg. of cystine-N, 0.03-0.04 mg. of S, 0.01 mg. of methionine-N by volatile iodide, and 0.05-0.1 mg. of methionine-N by homocysteine with a total of 2.5 ml. of 0.04N tetrathionate.

(2) 20% $CdCl_2$ and 20% $BaCl_2$ solution.

(3) Saturated $HgCl_2$ solution.

(4) 10% CH_3COOK solution in glacial acetic acid.

(5) KI. It should give no blank.

(6) N/100 $Na_2S_2O_3$.

(7) N/100 $KIO_3.HIO_3$.

(8) 25% CH_3COONa solution.

(9) N/10 $KIO_3.HIO_3$ and N/10 $Na_2S_2O_3$ for making tetrathionate solution which should be freshly prepared just before use by mixing at the rate of 2 ml. of N/10 $KIO_3.HIO_3$, 1 ml. of 10% HCl, a little KI and 2ml. of N/10 $Na_2S_2O_3$ and the end point is adjusted with N/100 $Na_2S_2O_3$ solution.

(d) PROCEDURES.-(1) Hydrolysis. About 0.5 g. of the grass protein preparation is weighed and transferred

to the digestion flask. The flask is filled with nitrogen gas which is washed with 5% $AgNO_3$ and concentrated H_2SO_4. A few small pieces of boiling chips, 10 ml. of HI and about 0.02 g. of potassium hypophosphite are added. The flask is then connected to the condenser which is kept at about 50°C. The absorption train, having been filled as described in the previous paragraph, is assembled and attached to the condenser. The digestion flask is placed in an oil bath kept at 150°C. The first three absorbers are immersed in warm water at about 50-60°C. During hydrolysis, the whole apparatus is aerated with nitrogen gas. A pinch-cock is attached to the side tube of the digestion flask to maintain a constant bubbling rate of nitrogen gas so that the bubbles can be counted. Boiling and aeration are continued for 5-6 hours for pure protein and for 7-8 hours for grass protein. At the end of the digestion, the absorption train is disconnected at the condenser outlet and the condenser jacket is drained. Another 0.02 g. of potassium hypophosphite is added to the digestion flask. Connection is then made with a pump at the condenser outlet and the digest is concentrated to about 3 ml. under reduced pressure to get rid of any iodine, the stream of nitrogen being continued.

(2) Dilution of the digest. The flask containing the digested protein is allowed to cool in a current of nitrogen and then it is disconnected. The digest is poured into a 25 ml. volumetric flask and the digestion flask is rinsed with 4% HCl which has been saturated with nitrogen. After making up to the volume, it is put aside to settle. During the digestion of impure leaf protein, a small amount of humin is produced and on the dilution of the concentrated digest, an orange coloured flocculum appears.

(3) Determination of methionine by volatile iodide. The 4th and 5th absorbers are rinsed into a 100 ml. volumetric flask containing 25 ml. of 25% sodium acetate solution. About 10-12 drops of formic acid (a little excess to reduce the bromine) are added, and the flask is whirled and diluted to the mark. Aliquots of 20 or 25 ml. are pipetted out and a little KI and a few drops of 10% HCl are added. The iodine liberated is titrated with N/100 $Na_2S_2O_3$ using starch solution as indicator. Methionine is calculated from the $Na_2S_2O_3$ used. One ml. of N/100 $Na_2S_2O_3$ is equal to 0.0234 mg. of methionine-N or 0.248 mg . of methionine.

(4) Determination of sulphur by H_2S and SO_2. The H_2S and SO_2 reduced from sulphate are absorbed in the

Cystine, m.w. 240.30. 11.66% of N

$$2\,SH\cdot CH_2\cdot CH(NH_2)\cdot COOH + I_2 \rightarrow \begin{matrix} S\cdot CH_2\cdot CH(NH_2)\cdot COOH \\ | \\ S\cdot CH_2\cdot CH(NH_2)\cdot COOH \end{matrix} + 2HI$$

N/100 $KIO_3 \cdot HIO_3$ equivalent to $\frac{2.40}{2} = 1.20$ mg. of cystine.

methionine, m.w. 149.21, 9.38% of N

$$CH_3COOK + I_2 + 6H_2O \rightarrow KIO_3\cdot HIO_3 + 2CO_2 + 7H_2$$

$$KIO_3\cdot HIO_3 + 11HCl + 10KI \rightarrow 6I_2 + 11KCl + 6H_2O$$

$$2Na_2S_2O_3 + I_2 \rightarrow Na_2S_4O_6 + 2NaI.$$

Since $\frac{1}{6}$ of the I liberated comes from $KIO_3\cdot HIO_3$,

N/100 $Na_2S_2O_3$ equivalent to $\frac{1.49}{6} = 0.248$ mg. of methionine

$$\underbrace{S\cdot CH_2\cdot CH_2\cdot \overset{NH}{\underset{|}{C}}H\cdot CO}_{\text{Lactone ring}} + H_2O \rightarrow SH\cdot CH_2\cdot CH_2\cdot \overset{NH_2}{C}H\cdot COOH$$

Homocysteine

$$2\,SH\cdot CH_2\cdot CH_2\cdot \overset{NH_2}{C}H\cdot COOH + I_2 \rightarrow$$

$$\begin{matrix} S\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH \\ | \\ S\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH \end{matrix} + 2HI$$

second absorber as CdS and $BaSO_3$ which can be oxidized by iodine to S and SO_4. The solution in the second absorber is rinsed into a beaker and a small excess of N/100 $KIO_3.HIO_3$ is added, followed by a few crystals of of KI and 2 ml. of 10% HCl. After standing for a little while, the excess iodine is titrated with N/100 $Na_2S_2O_3$ using starch indicator. One ml. of N/100 ~~$Na_2S_2O_3$~~ $KIO_3.HIO_3$ is equal to 0.16 mg. of sulphur.

(5) Determination of cystine and/or cysteine. From the diluted digest, two portions of 10 ml. are pipetted out into two 50 ml. conical flasks which have been filled with nitrogen. A few ml. of N/100 $KIO_3.HIO_3$ are added from a burette until the solution is definitely orange in colour. The excess iodine is titrated with N/100 $Na_2S_2O_3$. Since the digest is slightly yellow, the starch indicator should be added earlier than usual. When the end-point is approaching, the $Na_2S_2O_3$ solution should be added drop by drop very slowly so as to ensure a correct end-point. One ml. of N/100 $KIO_3.HIO_3$ is equivalent to 0.1399 mg. of cystine-nitrogen of 1.2 mg. of cystine.

(6) Determination of methionine by homocysteine. The digest left after the determination of cystine is used for determining methionine. The methionine has

been converted into the lactone of homocysteine which is not affected by iodine during the determination of cystine. When the cystine determination is finished, the digest is still retained in the conical flask. To the digest, a few drops of bromothymol blue are added together with one drop of caprylic alcohol and a quantity of freshly prepared 0.04N tetrathionate solution so as to give, with the tetrathionate produced during the cystine titration, an excess of 2.5 ml. One ml. of 0.04N tetrathionate is equivalent to 6 mg. of methionine, and the amount of methionine expected to be present is known from the volatile iodide determination. The flask is fitted with a rubber stopper, carrying two connecting tubes with rubber tubes. It is again filled with nitrogen gas. One of the connecting tubes is closed with a pinch-cock and the other is connected to the pump for deaeration. When the flask is evacuated, the tube is closed with another pinch-cock, leaving enough rubber tubing to insert a burette. Through the connecting tube, about 2 ml. of concentrated NH_4OH are drawn in until the digest shows a blue colour indicating it is definitely alkaline in reaction. The flask is evacuated again, close off, and immersed in warm water at 40°C. After standing in warm water for 15 minutes,

the flask is filled with nitrogen gas and both connecting tubes are opened, taken off and rinsed with water. The digest is acidified with 10 ml. of 5N HCl and the thiosulphate produced during the process of oxidation in the digest is titrated with N/100 $KIO_3.HIO_3$. To get a more satisfactory end-point, it is better to add a slight excess of the iodate and titrate back with N/100 $Na_2S_2O_3$. Methionine is calculated from the iodine consumed. One ml. of N/100 $KIO_3.HIO_3$ is equivalent to 0.1407 mg . of methionine-nitrogen or 1.49 mg. of methionine.

(e) DISCUSSION.-During the hydrolysis of protein, in order to prevent any I and HI coming over from the flask, the introduction of one more absorber containing red phosphorus suspension is recommended. Kuhn (1939), in his micromodification of Baernstein's method, also used red phosphorus for this purpose. Baernstein (1936) found that it was unnecessary to keep the absorbers warm, and Kassell and Brand (1938) found that the condenser need not be kept warm. But to prevent the detention of methyl iodide, it is advisable to keep both the condenser and the first three absorbers warm. Lugg (1938) recommended that the digestion of the protein be commenced with the addition of 0.02 g. of

KH_2PO_2, with addition of like amounts of KH_2PO_2 at intervals as digestion proceeds so long as the iodine coloration continues to develop, and that the digest be concentrated after hydrolysis with a final addition of 0.02 g. of KH_2PO_2. In hydrolysing impure leaf protein, however, the digest appeared to be dark orange in colour for reasons other than the presence of iodine making it impossible to judge the presence of iodine by its colour. It has been found to be sufficient to add two lots of 0.02 g. each of KH_2PO_2 before and after hydrolysis.

(f) DIAGRAMS SHOWING THE APPARATUS AND THE PROCEDURE FOR DETERMINING SULPHUR-CONTAINING AMINO ACIDS AND INORGANIC SULPHUR.-The arrangement of the apparatus and the different steps in the determination of sulphur-containing amino acids can be shown concisely in the following diagrams:

(1) Arrangement of apparatus.

Digestion flask	Absorber I	Absorber II	Absorber III	Absorber IV	Absorber V
0.5 g. of sample, 10 ml. of HI, 0.02 g. of KH_2PO_2, and a few boiling chips.	10 ml. 1% red P in water.	10 ml. sol. of 20% $CdCl_2$ and 20% $BaCl_2$.	10 ml. of sat. sol. of $HgCl_2$.	10 ml. of CH_3COOK in glac. acetic acid & 6 drops of Br.	5 ml. CH_3COOK in glac. acetic acid & 3 drops of Br.

(2) Procedure.

Protein

0.5 g. of sample is boiled for 5-6 or 7-8 hours at 150°C with 10 ml. of HI containing some KH_2PO_2 and a few boiling chips, and aerated with N.

(I) Methionine (II) Inorganic sulphate (III) Cystine

Methyl iodide — Lactone of homocysteine — H_2S and SO_2 — Cysteine

in 4th & 5th absorbers

Wash into 100 ml. flask containing 25 ml. of 25% CH_3COONa, remove Br with formic acid, & dilute to mark.

Aliqots of 20 or 25 ml. are treated with KI and HCl, and titrated with N/100 $Na_2S_2O_3$.

1 ml. of N/100 of $Na_2S_2O_3$ is equal to 0.0234 mg. of methionine-N or 0.248 mg. of methionine.

in 2nd absorber

Add some N/100 $KIO_3.HIO_3$, a few crystals of KI, & a few ml. of 10% HCl, excess of I is titrated with N/100 $Na_2S_2O_3$.

1 ml. of N/100 of $KIO_3.HIO_3$ is equal to 0.16 mg. of S.

in digestion flask

Wash into 25 ml. flask with 4% HCl and dilute to mark with water. Aliquots of 10 ml. are transferred to conical flasks filled with N.

Add a few ml. $KIO_3.HIO_3$. Excess I is titrated with N/100 $Na_2S_2O_3$.

1 ml. N/100 $KIO_3.HIO_3$ is equal to 0.1399 mg. to cystine-N or 1.2 mg. of cystine.

Add 2-3 drops bromothymol blue, 1 drop caprylic alcohol and a few ml. of fresh $Na_2S_4O_6$. The flask is evacuated. Add NH_4OH, & immered in warm water. Acidify with HCl. The thiosulphate is titrated with N/100 $KIO_3.HIO_3$.

1 ml. N/100 $KIO_3.HIO_3$ is equal to 0.1407 mg. of methionine-N or 1.49 mg. of methionine.

PRELIMINARY INVESTIGATIONS. (a) MODIFICATION OF THE METHOD.-In order to see whether it is necessary to introduce the red phosphorus absorber and to keep the condenser and the first three absorbers warm, several determinations were carried out in the following different ways; (1) no red phosphorus asborber, and the condenser and the first three absorbers were cold; (2) with red phosphorus absorber, and the condenser and the first three absorbers were cold; (3) no red phosphorus absorber, but the condenser and the first three absorbers were warm; and (4) with red phosphorus absorber, and the condenser and the first three absorbers were kept warm. B.D.H. edestin, containing 15.58% N, was used for this investigation, and the hydrolysis lasted for six hours. The results, from which the figures for blanks (see page 103) have been deducted, were as follows:

Table XXV.

Method	Sulphur as % of edstin					% distribution of S		
	Cystine	Methionine			SO_4	Cyst.	Meth.	SO_4
		V.I.	H.	Aver.				
(1)	0.393	0.442	0.438	0.440	0.028	45.64	51.10	3.26
(2)	0.388	0.435	0.442	0.439	0.029	45.33	51.28	3.39
(3)	0.385	0.460	0.438	0.449	0.028	44.65	52.09	3.26
(4)	0.388	0.446	0.438	0.442	0.027	45.25	51.58	3.17

V.I. = volatile iodide method.
H. = homocysteine method

As the above table shows, there is not much difference between the figures for cystine-S and the methionine-S (by homocysteine) got by the different methods. But the percentage of the methionine-S by volatile iodide differs with the different methods. The figures obtained by the second method is a little bit lower, and that by the third method is rather higher, than that by the fourth method. The detention of some volatile iodide by the cold condenser and the first three absorbers might be responsible for the low value in the former case; and the high value in the latter case might be attributable to the coming over of the I or HI from the digestion flask. The figures by the first method is quite close to that of the fourth method. It is probably due to a compensation of errors. On the whole it seems to be advisable to introduce a red phosphorus absorber and to keep the condenser and the first three absorbers warm, although the first method and the second method gave approximately the same results.

The figures given in Table XXV are expressed on a "sulphur basis", and the % distribution of sulphur was calculated on the assumption that cystine-S, methionine-S and SO_4 are the only three sulphur fractions

present in the protein. For the convenience of comparing with the figures found by other workers in analysing edestin, the results of the fourth method are converted into terms of nitorgen and listed below together with the results of the previous investigations.

Table XXVI.

Workers	Method	% N	% cyst.-nitrogen	% methionine-N		
				V.I.	H.	Aver.
Folin and Marenzi (1929)	Colorimetric uric acid reagent		@1.00			
Sullivan and Hess (1930)	Colorimetric Sullivan method		@0.89			
Baernstein (1932)	Gasometric		@1.31			
Baernstein (1936)	HI method			@1.40	@1.29	@1.35
Lugg (1938,1)	" "	18.50	0.90			1.18
" "	Differential oxidation	"	0.94			1.24
Present investigation	HI method	15.58	X1.09	X1.25	X1.23	X1.24

@ calculated from the figures which were originally reported as "% protein", assuming that the edestin sample contains 16% of nitrogen.
X corrected according to the C.F. on page 117.

On the whole, the results by different workers, as shown above, agree quite well. The figures for cystine-N vary from 0.89 to 1.31, and those for methionine-N from 1.18 to 1.35.

(b) ANALYSIS OF PURE SUBSTANCES.-In this investigation, B.D.H. cystine, methionine, and edestin were used either separately or mixed. Hydrolysis lasted for six hours, a red phosphorus absorber was inserted and the condenser and the first three absorbers were kept warm. The results, after deduction for the blank, are shown in the follwoing tables:

(1) Cystine.

Table XXVII.

No. of test	Percentage recovery	% S liberated as H_2S
1	98.4	1.01
2	96.2	1.54
3	97.2	1.48
4	98.0	0.98
Average	97.5	1.25

(2) Methionine.

Table XXVIII.

No. of test	% methionine-N as cystine-N	% recovery		% S liberated as H_2S
		V.I.	H.	
1	4.5	94.2	92.0	0.94
2	6.2	95.3	91.5	0.88
3	3.8	96.8	91.3	0.89
4	6.4	95.7	89.6	0.86
Average	5.2	95.5	91.1	0.89

(3) Cystine amd methionine. About equal amounts

of cystine and methionine were mixed for analysis.

Table XXIX.

No. of test	Percentage recovery		
	Cystine	Methionine	
		V.I.	H.
1	100.2	94.6	90.2
2	98.4	95.0	93.0
Average	99.3	94.8	91.6

(4) Edestin mixed with cystine and methionine. Portions of 0.51 g. and 0.48 g. of edestin were mixed with 10-15 mg. each of cystine and methionine and hydrolysed with 10 ml. of HI for six hours. For the purpose of ensuring accurate weighing of such minute quantities, about 0.1 g. each of cystine and methionine was dissolved in 10 ml. of HI separately, and 2 ml. portions of each solution were mixed with the edestin used for analysis. To the mixture, another 6 ml. of HI were added, making a total of 10 ml. The results, from which the amounts of cystine-N and methionine-N (as recorded in Table XXVI on page 113), in the edestin used have been deducted, are given in the table below:

Table XXX.

No. of test	Cystine-N (mg.)					Methionine-N (mg.)				
	T.	E.	D.	A.	%R.	T.	E.	D.	A.	% R.
1	2.50	0.87	1.63	1.66	98.2	(a) 2.24	0.99	1.25	1.30	96.1
						(b) 2.14	0.98	1.16	"	90.0
2	2.47	0.82	1.65	1.66	99.4	(a) 2.16	0.94	1.22	"	93.9
						(b) 2.13	0.92	1.21	"	93.0
Aver.					98.8	(a)				95.0
						(b)				91.5

T = total, E = edestin, D = difference, A = added, R = recovery, (a) for figures by the volatile method, and (b) for figures by the homocysteine method.

With regard to the recovery of pure substances, Baernstein (1936) got a very satisfactory result, namely 101.5% for cystine, 99.4% for methionine by the volatile iodide method, and 99.6% for methionine by the homocysteine method. That was not the case in the present investigation. The average recovery for cystine was 97.5%, for methionine by the homocysteine method it was 91.1%, and for methionine by the volatile iodide method it was 95.5%, when they were analysed separately. The recovery of cystine was quite good, the error being only 2.5%, which may be due partly to the decomposition of cystine into H_2S and partly to the unavoidable errors of technique. The low recovery of methionine as volatile iodide is due to the formation of methyl mercaptan, which, according to Kassell and Brand (1938), might amount to 3-4% of the methionine analysed. The

recovery of methionine as homocysteine was even lower than as methyl iodide, and a small quantity of cystine, which amounted to about 3.8-6.4% of the methionine-N (see table XXVIII on page 114), was found after deduction of the blank. This proves that a small proportion of the homocysteine was titrated as cystine, a point which is confirmed by the results in (3) page 115. When the analysis was carried out with a mixture of cystine and methionine, the recovery of cystine was higher than when the cystine was analysed alone, while the recovery of methionine both by the volatile iodide method and by the homocysteine method in (2) (see page 114) did not show any significant difference from that in (3). As a whole, the percentage recovery was in good agreement with that got by Kassell and Brand (1938). They got 97.8% for cystine, 93.7% for methionine by volatile iodide, and 89.3% for methionine by homocysteine.

For the subsequent analysis of grass protein preparations, correction factors were calculated. Since the grass protein preparations contain both cystine and methionine, the factor 1.007 was used for cystine according to the recovery in (3) on page 115, assuming that the protein contains approximately equal amounts of cystine and methionine. The correction factors for methionine by volatile iodide and by homocysteine were

1.047 and 1.098 respectively according to the recovery in (2) on page 114.

During the hydrolysis of cystine and methionine, a small portion of their sulphur was liberated in the form of H_2S. The percentage sulphur liberated as H_2S was 1.25% for cystine and 0.89% for methionine. The figures found by Kassell and Brand (1938) were about 2% for cystine and 1% in the case of methionine.

(c) TIME OF HYDROLYSIS.-Suitable portions of White clover protein preparation, sample 18, and mixtures of the same protein preparation with cystine and methionine were hydrolysed with HI for different lengths of time. It might be noted that the protein preparation of sample 18 contained about 5% ash, and that the ash contents of the protein preparations from grasses varied from about 4% to 8% in comparison with the figures of from about 1% to 14% got by Lugg (1938, 1, 2). The results of the different determinations were as follows:

(1) Grass protein preparation.

Table XXXI.

Hours	% Cyst.-N	% methionine-N V.I.	H.		% distribution of S Cyst.	Meth.	Others
4 hours	1.20	1.22	0.95	(a)	46.09	46.86	7.05
				(b)	49.51	42.91	7.58
5 hours	1.27	1.28	1.04	(a)	46.23	45.87	7.90
				(b)	50.22	41.13	8.65

6 hours	1.32	1.34	1.10	(a)45.30	46.00	8.70
				(b)49.37	41.15	9.48
7 hours	1.35	1.38	1.13	(a)44.87	45.84	9.29
				(b)48.92	40.94	10.14
8 hours	1.35	1.39	1.13	(a)44.65	45.96	9.39
				(b)48.85	40.56	10.59

(2) Grass protein mixed with cystine and methionine. The amounts of the cystine and methionine added were deducted according to their respective recoveries. The H_2S liberated from the added cystine and methionine was also deducted, but not from those originally present in the sample, at the rate of 1.25% from cystine and 0.89% from methionine as shown on page 114.

Table XXXII

Hours	% Cyst-N	% methionine-N V.I.	% methionine-N H.	% distribution of S Cyst.	Meth.	Others
4 hours	1.10	1.11	0.85	(a) 46.22	46.65	7.13
				(b) 52.02	40.09	7.89
5 hours	1.23	1.25	1.00	(a) 45.88	46.62	7.50
				(b) 50.59	41.14	9.27
6 hours	1.31	1.34	1.09	(a) 45.12	46.15	8.73
				(b) 49.37	41.07	9.56
7 hours	1.34	1.39	1.14	(a) 44.51	46.18	9.31
				(b) 48.56	41.30	10.14
8 hours	1.35	1.40	1.13	(a) 44.43	46.07	9.50
				(b) 48.75	40.82	10.43

(a) according to the methionine-N by volatile iodide.
(b) according to the methionine-N by homocysteine.

The figures in the above two tables agree well

Table XXXIII. Summary of the contents of S-containing amino acids.
(see Table V facing page 29 for particulars of the samples)

% of total-N in protein preparation as cystine- and/or cysteine-N				% of total-N in protein preparation as methionine-N							
				by volatile iodide				by homocysteine			
W.C.	C.	R.	T.	W.C.	C.	R.	T.	W.C.	C.	R.	T.
1.18				1.22				1.04			
1.33				1.40				1.15			
1.35				1.38				1.13			
1.01				1.20				0.90			
1.14		1.16		1.26		1.30		1.02		1·10	
1.20	1.01	1.12		1.42	1.21	1.52		1.11	0.73	1.01	
		0.96				1.40				1.05	
1.10	1.25	1.10		1.23	1.40	1.18		0.98	1.08	1.00	
1.32		1.16		1.50		1.50		1.20		1.06	
		1.03				1.28				0.97	
		0.98				1.34				0.89	
1.40	1.46	1.26		1.46	1.30	1.43		1.14	1.22	1.10	
1.36	1.06	1.08		1.48	1.41	1.14		1.20	0.84	0.95	
	1.34	1.03			1.28	1.20			1.07	0.94	
1.03				1.30				0.94			
	1.37				1.52				1.12		
	1.42				1.46				1.24		
	1.13				1.35				1.03		
	0.91				1.29				0.98		
	1.12				1.30				1.03		
	1.42				1.54				1.21		
	1.16				1.50				1.09		
	1.44				1.46				1.15		
	1.28				1.59				1.17		
	1.32				1.52				1.20		
	1.25				1.30				0.84		
		1.04				1.27				1.04	
		1.21				1.55				1.10	
		1.15				1.25				1.05	
0.90		1.22		1.29		1.08		0.70		0.95	
1.04		1.02		1.32		1.22		1.00		0.94	
		1.16				1.43				0.91	
			1.13				1.17				0.9
			1.24				1.53				1.
			0.85				1.40				0.
			1.25				1.26				1.
			1.32				1.59				1.
			1.20				1.38				1.
			0.86				1.04				0.
			0.82				1.34				0.

and show that seven hours hydrolysis seems to be sufficient to produce the maximum yield. The yield of methionine-N by the iodide method is always about 25% higher than that by the homocysteine method. Since all the figures have been corrected, the cause of the abnormally high yield of methionine-N by volatile iodide probably lies in the fact that the protein preparation is not entirely free from impurities which might evolve some volatile iodide.

RESULTS AND DISCUSSION. The contents of sulphur-containing amino acids of the grass protein preparations are recorded in Table XXXIII. They are all reported on a "N basis", namely the nitrogen in cystine and methionine as % of the nitrogen in the protein preparations. All the figures were corrected according to their respective correction factors, i.e. 1.007 for cystine, 1.047 for methionine by volatile iodide, and 1.098 for methionine by homocysteine. The figures for methionine by volatile iodide are, in all cases, higher than those by homocysteine as explained in the previous paragraph. The sulphur-contents of cystine, methionine, and inorganic-sulphur are expressed as "% distribution of sulphur" on the assumption that cystine and methionine are the only two sulphur-containing amino acids and

all the other forms of sulphur are in the state of sulphate. The % distribution of sulphur is calculated according to the methionine by volatile iodide and by homocysteine separately and is given in Tables XXXVII and XXXVIII. The figures for the % distribution of sulphur have not been corrected, because the amount of H_2S liberated from cystine and methionine is negligible, being only 1.25% of the cystine-S and 0.89% of the methionine-S. In view of the ever present danger of high results by volatile iodide, the figures for methionine by homocysteine would seem to be more reliable.

The figures of cystine and methionine, as reported in Table XXXIII, do not show any noteworthy differences among the four species. The figures for cystine- and/or cysteine-N vary from 0.90-1.40% in White clover, from 0.91-1.46% in Cocksfoot, from 0.96-1.26% in Rye-grass, and from 0.82-1.32% in Timothy. The figures for methionine-N by homcysteine lie within the range 0.70-1.20% in White clover, 0.73-1.24% in Cocksfoot, 0.91-1.10% in Rye-grass, and 0.74-1.29% in Timothy. Lugg (1938, 1) analysed ten samples of grass protein preparations and found that cystine- and/or cysteine-N contents varied from 1.1-1.7% and the methionine-N from 1.2-1.6%. He also found that Lucerne leaves had slightly lower cystine and methionine contents than other leaf proteins,

which was contrary to the finding of Pollard and Chibnall (1934). They reported that Lucerne leaf protein was particularly rich in cystine compared with grass proteins. In the present investigation, the figures are invariably lower than those obtained by Lugg; and White clover, though of the same family as Lucerne, contained about the same amounts of cystine and methionine as the grasses.

In considering the effect of the stage of growth, Lugg (1938, 2) reported that the sulphur distribution of leaf protein provided no evidence of variation with the age of leaves. But according to the results of this investigation, the stage of growth did exert some effect on the contents of sulphur-containing amino-acids, the cystine and methionine showing the similar variations. Generally speaking, young and grazed samples contained less cystine and methionine than the older ones, and the maximum contents of cystine and methionine were reached at the stage of flowering or heading to ripening. This general statement holds true for all four species with one exception, where the cystine-N in Cocksfoot, sample 58, which was taken in July 1940, was only 0.91%, being the lowest figure for all the Cocksfoot samples. The results for methionine-N by

Table XXXIV. Summary of the contents of S-containing amino acids in grasses under different manurial treatments. (see Table VI facing page 29 for particulars of samples)

% of total-N in protein preparation as cystine- and/or cysteine-N	% of total-N in protein preparation as methionine-N	
	V.I.	II.
Cocksfoot		
0.93	1.34	0.84
1.09	1.34	0.94
Rye-grass		
1.33	1.38	1.04
1.16	1.36	1.01
Cocksfoot		
1.00	1.12	0.81
0.96	1.20	0.99
Rye-grass		
1.03	1.04	0.93
1.04	1.19	0.99
Rye-grass		
1.08	1.34	0.96
0.94	1.27	0.94

volatile iodide were rather irregular due presumably to the distubing effect of the volatile iodide from some extraneous substances.

The different manurial treatments had no important effects on the percentages of cystine-N and methionine-N, and the results, as shown in Table XXXIV, were rather irregular. The application of slag tended to increase the percentage of cystine-N and methionine-N in Cocksfoot, and to decrease the percentage of these two amino acids, especially cystine, in Rye-grass. Lime appeared to cause a slight increase in the percentage of sulphur-containing amino acids in both Cocksfoot and Rye-grass except that the cystine content of Cocksfoot was lower in the sample from the high lime plot. Ammonium sulphate reduced the percentage of both cystine and methionine in Rye-grass, but its effect was not very large.

Concerning the effect of season on the percentage of cystine-N and methionine-N, it appears that White clover and the grasses did not follow the same trend. As summarized in Table XXXV, the figures for White clover reached their maximum in July and decreased gradually afterwards. The seasonal variation for the grasses were very similar among themselves but different from White clover; after having reached their maximum in July,

Table XXXV.

Month	% of total-N in protein preparation as cystine- and/or cysteine-N			
	W.C.	C.	E.	T.
May	---	1.28-1.32	---	---
June	0.90-1.32	1.25-1.42	1.04-1.16	0.86 — 1.25 ~~0.78-1.06~~
July	1.33-1.40	1.42-1.46	1.21-1.26	1.24 — 1.32 ~~1.19-1.29~~
August	1.20-1.36	1.05-1.16	1.03-1.12	0.82 ~~0.84~~
September	1.01-1.10	1.12-1.44	0.98-1.10	0.85 — 1.20 ~~0.87-1.12~~
October	---	1.34	1.03-1.15	---
	% of total-N in protein preparation as methionine-N (by homocysteine method)			
May	---	1.17-1.20	---	---
June	0.70-1.20	0.84-1.21	0.91-1.10	0.78-1.06
July	1.13-1.15	0.98-1.24	1.10-1.10	1.19-1.29
August	0.94-1.20	0.73-1.09	0.95-1.05	0.74
September	0.90-1.00	1.03-1.15	0.89-1.00	0.97-1.12
October	---	1.07	0.94-1.05	---

there was a general tendency for a decline in August or September and a rise in September or October. This statement applies to both cystine-N and methionine-N with one exception, where a figure for a Cocksfoot sample, which was sampled in July 1940, was exceptionally low and has not been listed in the above table. The difference in seasonal variation between White clover and grasses may be explained by the fact that the growing period of White clover is somewhat restricted, whereas the grasses have a longer period of growth.

A study of the contents of amino-N and the N in S-containing amino acids will reveal that there is some correlation between these constituents. For the purpose of investigating their relationship, the figures for amino-N and those of the nitrogen in S-containing amino acids for all 63 samples are summarized as follows:

Table XXXVI.

Sample No.	% protein-N as amino-N	% protein-N as S-amino acids	Sample No.	% protein-N as amino-N	% protein-N as S-amino acids
1	66.4	2.22	33	64.1	2.59
2	60.0	2.49	34	59.3	1.87
3	60.3	2.08	35	67.5	2.08
4	64.1	2.04	36	64.2	2.33
5	64.6	2.16	37	58.2	2.10
6	61.1	2.26	38	70.2	2.41
7	57.6	1.64	39	61.6	1.97
8	58.6	2.52	40	62.8	2.20
9	63.2	2.63	41	61.2	2.45
10	66.1	2.22	42	62.3	2.52
11	62.9	2.54	43	62.6	2.09
12	71.8	2.68	44	60.2	2.07
13	68.0	2.36	45	58.0	2.17
14	71.2	2.48	46	62.3	2.31
15	63.2	2.66	47	60.6	1.60
16	63.4	2.31	48	58.3	1.77
17	66.5	2.43	49	59.0	2.03
18	73.0	2.48	50	63.1	2.42
19	67.8	2.31	51	54.8	2.17
20	63.6	1.74	52	60.3	1.81
21	53.2	2.13	53	62.3	1.95
22	57.2	2.01	54	57.3	1.96
23	56.8	1.56	55	63.3	2.03
24	62.4	2.25	56	64.7	2.04
25	59.2	2.00	57	56.7	1.88
26	68.4	1.97	58	57.3	1.89
27	71.7	2.56	59	62.4	2.61
28	60.7	1.89	60	69.6	2.04
29	57.3	2.03	61	62.8	2.15
30	65.5	1.91	62	62.2	1.96
31	63.0	2.16	63	56.9	2.32
32	62.4	1.82			

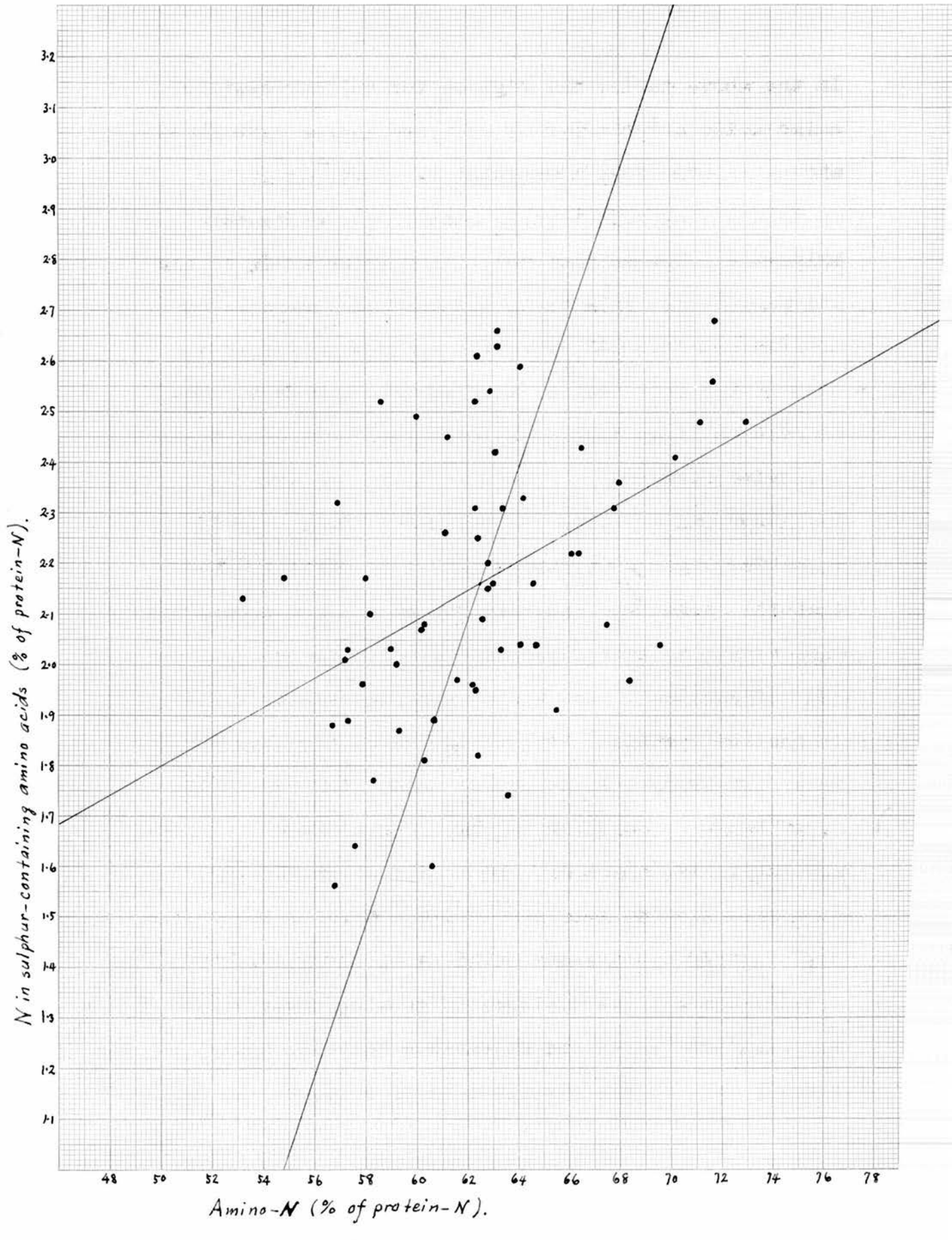
Table XXXVII. Summary of % distribution of S (according to the Methionine-by homocysteine, see Table V facing page 29 for particulars of samples)

White clover			Cocksfoot			Rye-grass			Timothy		
Cyst.	Meth.	Others	Cyst.	Meth.	Others	Cyst.	Meth.	Others	Cyst.	Meth.	Other
43.41	38.26	18.33									
47.89	41.41	10.70									
48.92	40.94	10.14									
37.33	33.27	29.40									
36.55	34.01	29.44				47.06	43.00	9.94			
46.49	43.01	10.50	45.85	33.13	21.02	47.69	43.00	9.31			
						39.38	43.06	17.56			
39.33	35.04	25.63	48.07	41.52	10.41	43.06	39.14	17.80			
45.58	41.44	12.98				46.82	42.78	10.40			
						43.22	41.29	15.39			
						40.48	36.76	22.76			
47.59	38.75	13.66	48.90	40.86	10.24	47.73	41.67	10.60			
46.60	41.12	12.28	44.44	35.60	19.96	41.18	36.23	22.59			
			49.74	39.71	10.55	44.05	40.22	15.73			
41.05	37.42	21.53									
			45.44	37.15	17.41						
			45.85	40.03	14.12						
			41.41	37.76	20.83						
			40.47	43.58	15,95						
			41.54	38.20	20.26						
			47.24	40.26	12.50						
			42.48	39.92	17.60						
			49.02	39.15	11.93						
			41.87	38.21	19.92						
			43.29	39.36	17.35						
			54.66	36.73	8.61						
						36.28	36.28	27.44			
						47.68	43.36	8.96			
						42.64	38.94	18.42			
40.69	31.58	27.73				50.88	39.63	9.49			
40.49	39.00	20.51				42.71	39.36	17.93			
						51.77	40.62	7.61			
									49.65	40.00	1(
									46.67	44.78	8
									37.37	42.64	1!
									44.65	37.86	1
									43.78	43.08	1
									43.39	40.49	1
									38.79	35.18	2
									39.34	35.50	2

Table XXXVIII. Summary of % distribution of S (according to the methionine by volatile iodide, see Table V facing page 29 for particulars of samples

White clover			Cocksfoot			Rye-grass			Timothy		
Cyst.	Meth.	Others	Cyst.	Meth.	Others	Cyst,	Meth.	Others	Cyst.	Meth.	Othe:
40.72	42.09	17.19									
43.93	46.24	9.83									
44.87	45.84	9.29									
33.66	39.93	26.41									
33.84	38.90	27.26				42.94	48.06	9.00			
41.51	49.12	9.37	37.64	45.09	17.27	39.18	53.17	7.65			
						34.43	50.21	15.36			
36.10	40.37	23.53	42.81	47.94	9.25	40.23	43.15	16.62			
41.30	46.93	11.77				39.75	52.01	8.24			
						38.35	47.65	14.00			
						34.13	46.68	19.19			
42.92	44.76	12.32	47.65	42.43	9.93	41.81	48.90	9.29			
42.52	46.27	11.21	35.85	48.15	16.00	38.37	40.51	21.12			
			46.29	44.08	9.63	39.66	46.20	14.14			
35.85	45.28	18.87									
			40.15	44.51	15.37						
			42.82	44.01	13.17						
			37.11	44.27	18.62						
			35.57	50.43	14.00						
			37.76	43.82	18.42						
			42.56	46.16	11.28						
			36.93	47.76	15.31						
			44.28	44.95	10.77						
			36.77	45.66	17.57						
			39.18	45.12	15.70						
			45.35	47.32	7.33						
						33.57	41.04	25.39			
						38.23	53.86	8.91			
						39.68	43.14	17.18			
32.06	45.97	21.97				48.27	42.75	8.98			
36.00	45.69	18.31				38.23	45.88	15.89			
						42.08	50.18	7.74			
									44.56	46.14	
									41.37	51.46	
									31.43	51.76	1
									41.67	42.01	1
									40.06	48.26	1
									39.37	45.60	1
									34.72	41.99	2
									30.55	49.92	:

Fig. X. Correlation between amino-N and the N in S-containing amino acids.



In the above table, the figures for sulphur-containing amino acids are the sum of cystine- and/or cysteine-N and methionine-N by homocysteine. There is little mention of the relationship between the contents of amino-N and the sulphur-containing amino acids in the literature. In this investigation, the correlation coefficient is +0.45, which is quite significant in view of the fact that 63 samples were analysed. The regression lines in Fig. X were drawn according to the following formulas:

(1) $A = 7.02S + 47.34$, (2) $S = 0.0288A + 0.36$

The figures in Tables XXXVII and XXXVIII show that the percentage distribution of sulphur showed the same variation as the percentage contents of cystine-N and methionine-N. The higher the figures for the percent of cystine-N and methionine-N, the more sulphur is in the form of amino acids. Since the percentage distribution of sulphur and the percentage of cystine-N and methionine-N are different ways of expressing approximately the same results, the observation on the variation in cystine and methionine, among different species, at different stages of growth, under various manurial treatments and at various seasons, apply equally well to the distribution of sulphur. One

striking point of the results on sulphur distribution is that, in all cases, the figures for sulphur in other forms than cystine and methionine were high when the samples were young, decreased gradually towards the stage of flowering or heading until the herbages were getting ripe, and rose again for the aftermath.

SUMMARY

For the present investigation, four of the most common species of forage crops, namely White clover, Danish Cocksfoot, Ayrshire Perennial Rye-grass, and Scotch Timothy, were selected. During the course of 1939 and 1940, sixty three samples were collected, from several farms, at various stages of growth, and under different manurial treatments for a study of the effects of different factors. The water content and carotene in the fresh samples, the total nitrogen in the dried samples, and the amino-N, sulphur-containing amino acids, and sulhpate sulphur in the protein preparations, were determined; and various methods of analysis were examined.

WATER CONTENT AND TOTAL NITROGEN. Generally, the water content decreased as the grass was getting old, and White clover, being less stemmy, contained more moisture than the other three species. Since the moisture content depends largely upon the climatic conditions prevailing when the samples are taken, it is not of great importance in discussing the results.

The total nitrogen content tended to fall as the plant reached and passed the flowering stage and then rose again for the aftermath. The rise in total nitrogen

in the aftermath was more pronounced as a result of grazing. White clover contained two or three times as much nitrogen as the grasses. The three species of grass contained about the same amount of nitrogen. In one area, slag tended to increase the nitrogen content in Rye-grass and to decrease the nitrogen content in Cocksfoot; lime reduced the nitrogen content of both Cocksfoot and Rye-grass; In another area, a late application of ammonium sulphate produced an increase of 30% in the nitrogen of Rye-grass ~~of 30%~~. All the samples were collected from May to October. During this period, the nitrogen content was high in May and June, decreased during July and August, and rose again in September and October.

CAROTENE. The carotene content of White clover, varying from 142 p.p.m. to 552 p.p.m., was much greater than that of the other three species. The figures for the grasses varied from 42 p.p.m. to 385 p.p.m. for Cocksfoot, from 4 p.p.m. to 261 p.p.m. for Rye-grass, and from 122 p.p.m. to 275 p.p.m. for Timothy.

With respect to the effect of the stage of growth, all four species showed a similar trend of variation. The carotene was greatest in the young plant, tended to decline at flowering or heading, decreased rapidly with fading or ripening, and rose again for the after-

math especially after grazing. The carotene content of White clover and Timothy seemed to be less affected by age than Cocksfoot and Rye-grass.

The effect of ammonium suphate was very remarkable, producing an increase of 25-35% over the control. Both slag and lime caused some increase of carotene, but their effects were not striking.

During different seasons the carotene contents of all four species were high in May and June, fell in July and August, and increased again in September and October.

The amounts of carotene in the heads or flowers were lower than in the stems and leaves except in the case of Rye-grass and in a few samples of Cocksfoot, where the reverse was the case.

The figures for total nitrogen showed a very close correlation with the carotene content. The correlation coefficient for 63 samples is +0.85 which means that there is no doubt about the high degree of association between carotene and total nitrogen.

PROTEIN PREPARATION. The extent of extraction varied from 15% to 43% according to the species of herbage and the conditions of the sample. White clover, being less fibrous, produced more protein preparation than

the grasses; and within each species, young and aftermath samples produced more protein preparations than the old ones. A few samples were subjected to a second extraction, and the amino nitrogen contents in the second protein preparations were determined. There seemed to be no striking difference between the protein first extracted and the protein left in the residue. The nitrogen content of the protein preparation was very irregular, varying from 6.1% to 14.1%, irrespective of the conditions of the sample. It depended more on the process of extraction than upon the origin of the protein.

AMINO-NITROGEN. There were no striking differences among the four species with regard to the amino-nitrogen contents of the protein preparations. Within each species, the percentage of amino-N tended to increase as the plant was getting ripe. Aftermath contained more amino-N than those samples at about the same stage of growth but taken earlier in the season from the same field. The effects of manurial treatments were variable. Slag increased the amino-N percentage in Cocksfoot and decreased it in Rye-grass. Lime increased it in both Cocksfoot and Rye-grass. Ammonium sulphate, on the other hand, decreased the percentage of amino-N in

Rye-grass. As to the effect of season, the amino-N of all four species was low in May and June, reached a maximum in July and dropped a little thereafter.

SULPHUR-CONTAINING AMINO ACIDS AND SULPHATE SULPHUR.

In view of the ever present danger of high results for methionine-N by the methyl iodide method due probably to the fact that the protein preparations are not entirely free from substances which might evolve some volatile iodide, the figures for methionine-N by homocysteine seemed to be more reliable.

The four species of herbage did not show significant differences in the sulphur-containing amino acids. The figures expressed as cystine- and/or cysteine-N varied from 0.82-1.46%, and those as methionine-N by homocysteine lay within the range 0.70-1.29%.

With respect to the effect of stage of growth, cystine and methionine showed a similar variation in all four species. Young and grazed samples contained less cystine and methionine than the old ones, and the maximum content of these two amino acids was reached at the stage of flowering or heading to ripening.

The effects of fertilizers were rather irregular and not very marked.

In regard seasonal variation, White clover did not show the same variation as the other species. The

figures for White clover, of which the growing period is somewhat restricted, reahced a maximum in July and decreased gradually afterwards. The grasses have a longer period of growth, and their cystine and methionine contents reached a maximum in July, fell in August or September, and rose again in September or October.

In calculating the percentage distribution of sulphur, it was assumed that cystine and methionine are the only two sulphur-containing amino acids and that all the sulphur in other forms is present as SO_4. The percentage distribution of sulphur followed the same variation as the percentage contents of cystine-N and methionine-N, and the observations on the variation in cystine and methionine contents under different conditions apply equally well to the distribution of sulphur. One point of interest is that the sulphur in the form other than cystine and methionine, was higher in young and grazed samples than in the older plants, leading to the conclusion that as the plant developed, the inorganic sulphur was converted into organic compounds.

A smaller but nevertheless quite significant correlation was foung between the contents of amino-N and sulphur-containing amino acids. The correlation coefficient is +0.45 for 63 samples.

OTHER REMARKS. In all cases, the effect of locality was not significant. Since the chemical composition of grass varied more according to the stage of growth than with other factors, and the samples taken from different farms were not exactly at the same stage of growth, it was impossible to compare the influence of different ~~factors~~ local conditions.

Generally speaking, the trend of the variation of the constituents during different seasons appeared to be in accordance with the stage of growth, and the effects of these two factors seemed to overlap each other.

One of the most striking features of the results was the beneficial effect of grazing, especially with respect to the nitrogen and carotene contents. This applied to all four species and at all stages of growth.

The ash-contents of the protein preparations varied from about 4% to 8% which may be regarded as fairly satisfactory for an extraction of this nature.

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